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Effects of branched-chain amino acid supplementation in dams fed a low-protein diet on protein metabolism of dams and liver development of offspring

어미 마우스의 분지아미노산 보충 섭취가 저단백 식이를 섭취한 어미의 단백질 대사와 자손의 간 발달에 미치는 영향

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Abstract

Effects of branched-chain amino acid supplementation in dams fed a low-protein diet on protein metabolism of dams and liver development of offspring

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Maternal nutritional environment is closely related to the growth of offspring. Maternal low-protein (LP) diet has been reported to cause catabolic state of dams and restrict fetal liver development, resulting in adult metabolic disease. Branched-chain amino acid (BCAA) can directly activate mTOR pathway and promote protein synthesis especially by leucine. Therefore, this study investigated whether maternal BCAA supplementation would alter maternal protein metabolism and liver growth of weaned offspring. Female CD-1 mice were fed a diet containing normal protein (NP, 20% casein), low protein (LP, 10% casein), LP with 2.1% BCAA (BCAA) or LP with 2.1% alanine (Ala) for two weeks before mating and throughout pregnancy and lactation. Dams and offspring were analyzed at postpartum (PP) day 21. To see whether maternal LP model was properly induced and maintained during pregnancy, dams and offspring were additionally analyzed at PP2 or 3. There were no significant

differences by BCAA supplementation in organ weights and serum parameters in dams and offspring at PP2 or 3. As compared to LP group, BCAA and Ala group of PP21 dams showed a significantly higher serum albumin, hepatic total protein and S6 protein levels which were significantly lower in LP group than in NP group. Moreover, as compared to NP group, BCAA and Ala group of PP21 dams showed the significantly higher leptin levels, which had a significant correlation with total liver protein levels, suggesting the relation between increased leptin and hepatic protein synthesis. PD21 offspring from dams fed an LP diet represented the features of retarded liver development, including higher proliferating cell nuclear antigen levels, lower levels of area of hepatic sinusoids, hepatocyte nuclear factor 4α which is core hepatic growth factor and low-density lipoprotein receptor which located on liver sinusoids. Both in BCAA-O and Ala-O group, area of sinusoids was significantly increased than in LP-O group. In conclusion, maternal BCAA supplementation from pre-pregnancy to lactation did not show specific effects on maternal protein metabolism and liver development of weaned offspring which were restricted by maternal LP diet. Improved indicators observed both in BCAA and Ala group of dams and offspring may have been induced by increased nitrogen of amino acid supplementation on LP diet, not by BCAA.

Keyword: BCAA supplementation, maternal low protein diet, protein metabolism, liver development, mice offspring

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List of Abbreviations

BCAA: branched-chain amino acid

BUN: blood urea nitrogen

COL1A1: collagen type 1 alpha 1

EAA: essential amino acid

HGF: hepatocyte growth factor

HNF4α: hepatocyte nuclear factor 4 alpha

LDLR: low-density lipoprotein receptor

mTOR: mammalian target of rapamycin

NEAA: non-essential amino acid

PCNA: proliferating cell nuclear antigen

α-SMA: alpha-smooth muscle actin

SR-B1: scavenger receptor B1

STAB2: stabilin-2

TC: total cholesterol

TG: triacylglycerol

VEGF-A: vascular endothelial growth factor-A

1. Introduction

1.1. Maternal nutritional environment and low-protein model

Nutritional status during the fetal or neonatal periods play a role in overall homeostatic regulatory mechanisms, thereby pool perinatal nutrition can cause intrauterine growth restriction (IUGR) and increase susceptibility to certain diseases in adulthood (Gluckman *et al.*, 2008). IUGR Fetuses have been reported to represent adaption such as reduced body size and reprogrammed metabolism for survival, but these fetal alteration results in disproportional growth and metabolic diseases in later life (Barker, 2004; Reichetzeder *et al.*, 2016b). Even minor modification of maternal diet during periconceptional period can change the fetal blood pressure and metabolism (Kwong *et al.*, 2000; Langley-Evans *et al.*, 1996).

Maternal low-protein (LP) diet is one of the well-established IUGR animal model. LP diet makes fetus divert nutrients to critical organs such as the brain to properly use a limited nutrient supply (Desai *et al.*, 1997). In other words, maternal LP diet disturbs the balanced organ development, especially in liver and pancreas rather than brain and lung (Desai *et al.*, 1995). Maternal LP diet has been reported to alter the glucose (Ozanne *et al.*, 1999; Shepherd *et al.*, 1997) and lipids metabolism (Burdge *et al.*, 2004; Sohi *et al.*, 2011) in fetus and adult offspring, and these metabolic changes may be associated with perinatal impairment of metabolic organs including

liver. Meanwhile, the timing of exposure to maternal diet is critical to determine the phenotype of offspring (Jahan-Mihan *et al.*, 2015). Preimplantational LP diet of dams can inhibit proliferation of blastocyst and induce the hypertension in adult (Kwong *et al.*, 2000). Also, maternal LP diet during gestation and/or lactation period structurally and functionally restricts the development of organ, including liver (Ramadan *et al.*, 2013), pancreas (Alejandro *et al.*, 2014) and kidneys (Costa *et al.*, 2015). However, maternal response to LP diet during pregnancy or lactation is a little different. Gestational LP diet reduces the anabolic hormones such as insulin, IGF-1 and leptin in dams (Jansson *et al.*, 2006). In this regard, sustained LP diet during pregnancy and lactation causes more intensified catabolic state such as decreased total protein and albumin levels, which are not observed in dams fed an LP diet during only pregnancy (Cherala *et al.*, 2006). In addition, growth of offspring from dams fed an LP diet during lactation were restricted although their dams consumed normal protein diet during pregnancy (Agnoux *et al.*, 2015; Zambrano *et al.*, 2006). Overall, the influences of maternal LP diet during pregnancy may be especially aggravated by the availability of protein during lactation (Bautista *et al.*, 2008) both in dams and offspring.

1.2. Effects of low-protein diet on maternal metabolic state

Pregnant dams undergo numerous metabolic changes to maintain adequate supply of nutrient to fetus, but LP diet consumption inhibits these normal adaptive response (Wang *et al.*, 2015). Several studies in human and animals have demonstrated that the common acute response to LP diet is a reduced protein turnover. Growing rat fed LP diet for 15 days showed decrease of protein synthesis and proteolysis in soleus muscle (Batistela *et al.*, 2014). Moreover, maternal LP diet decreases the urea synthesis (Holness, 1999). Reduced urea synthesis observed in animal fed LP diet seems to be resulted from up-regulated hepatic peroxisome proliferator-activated receptor alpha (Kalhan, 2016), and may be an adaptive response for conservation of nitrogen in restriction of available dietary amino acids (Kalhan, 2016). Lower blood urea nitrogen (BUN) (Cherala *et al.*, 2006) and urea (Parimi *et al.*, 2004) levels observed in dams fed an LP diet during pregnancy suggest that dams does not response differently from common animals to LP diet. Amino acid is an essential substrate for offspring growth, because amino acid is not only the building block of protein synthesis but also a signal messengers of cellular metabolism (Wu, 2009). In other words, maternal LP diet can affect growth of offspring via altered amino acids profile and nitrogen balance. Normal pregnancy induces the reduction of protein turnover and hypoaminoacidemia (Kalhan, 2000), but maternal LP diet exaggerates these normal adaptive response through alteration of essential amino acid (EAA) levels (Parimi *et al.*, 2004) and breaks the

balance of protein metabolism of dams. Decreased serum EAAs, especially branched chain amino acids (BCAA) levels, is the prominent feature of dams fed an LP diet during pregnancy. Threonine and histidine also have been reported to be reduced in plasma of LP dams (Rees *et al.*, 1999; Wang *et al.*, 2015). BCAA most sensitively responds to maternal LP diet and is reduced (Kwong *et al.*, 2000; Parimi *et al.*, 2004). Low maternal serum EAA levels decreases the activity of placental amino acid transporters through down-regulated mammalian target of rapamycin (mTOR) signaling (Jansson *et al.*, 2006), suggesting the lack of amino acids supply to fetus.

Maternal LP diet during pregnancy has been reported to decrease anabolic hormones such as insulin, IGF-1 and leptin in dams (Jansson *et al.*, 2006). Especially, leptin is reduced in maternal serum, amniotic fluids and serum of fetus by maternal LP diet (Starr *et al.*, 2015). Leptin acts on whole-body peripheral tissues, regulating glucose metabolism, insulin sensitivity, energy intake (Guilloteau *et al.*, 2009). Fetus or offspring can be exposed to leptin from various sources, including placenta, milk and endogenous leptin of offspring, and these leptin are important for the organ development of fetal and offspring (Vickers *et al.*, 2012). Thus, decrease of maternal leptin induced by consumption of LP diet can restrict growth and development of fetal and offspring.

1.3. Effects of maternal low-protein diet on liver development

The redistribution of organ development is the one of the fundamental mechanism that IUGR restricts the growth of fetus (Reichetzeder *et al.*, 2016a). In offspring from LP-fed dams, development of brain, heart and adrenal gland is given priority, but liver, kidney, pancreas and skeletal muscle are relatively sacrificed (Desai *et al.*, 1996). As a central site of metabolic homeostasis, especially, restriction of prenatal and postnatal liver development can cause disturbance of metabolism offspring in early and later life. Maternal LP diet has been reported to interfere the liver development structurally and functionally (Burns *et al.*, 1997), and these alteration may be related to development of liver disease in later life (Kwong *et al.*, 2006). Impaired liver growth and function increases the risk of cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease (Cianfarani *et al.*, 2012).

Prior to these functional changes, structural changes can be used as a more fundamental indicator of the inhibition of liver development by maternal diet. Maternal LP diet has been reported to decrease the absolute weight and relative weight of fetal liver (Rees *et al.*, 1999; Starr *et al.*, 2015). Abnormal cytoplasmic vacuoles and cellular structure accompanying lower hepatic proliferation and higher apoptosis as compared to control group were observed in liver of fetuses from dams fed an LP diet during pregnancy (Ramadan *et al.*, 2013). In the same study, LP fetuses showed significantly decreased total protein and increased glycogen levels in liver

compared with liver from fetuses of control group (Ramadan *et al.*, 2013), suggesting that biochemical changes may also be accompanied by structural changes in early liver.

Several studies have also reported that the effects of maternal LP diet on liver development in aspects of liver function, especially altered expression of gene and transcript factor (Vaiman *et al.*, 2011). Maternal LP diet during pregnancy decreased cholesterol 7 alpha-hydroxylase, hepatocyte nuclear factor 4 alpha (HFN4α) levels with increased tumor necrosis factor-alpha in liver of weaned offspring, resulting in increase of cholesterol levels in liver of adult offspring (Sohi *et al.*, 2011). Since reduction of hepatic cholesterol 7 alpha-hydroxylase levels is related to occurrence of arteriosclerosis and hypercholesterolemia, maternal LP may reprogram early hepatic cholesterol metabolism and increase the risk of cardiovascular diseases in adult (Sohi *et al.*, 2011). In addition, maternal LP diet during pregnancy and lactation increased peroxisome proliferator-activated receptor gamma signaling without change of hepatic lipids levels in liver of weaned offspring, suggesting that abnormal glucose and lipid metabolism can be already induced at weaning and it can disturbs the lipid metabolism in adult (Zheng *et al.*, 2015). Based on these results, maternal LP diet sustained from pregnancy to lactation may alter hepatic gene expression and increases susceptibility to adult metabolic disease.

1.4. Benefits of branched-chain amino acid supplementation

BCAA is a generic term for three EAA, leucine, isoleucine and valine (Shimomura *et al.*, 2004). Leucine is known to directly activate mTOR signaling pathway in liver and skeletal muscle, thereby promoting protein synthesis (Nair *et al.*, 2005; Nishitani *et al.*, 2004). Isoleucine and valine balance the BCAA levels in the body (Wu, 2009), and also contribute to activation of mTOR signaling as an amino acid. Because supplementation of leucine alone can cause imbalance of BCAA or no significant effects on increase of protein synthesis, BCAA rather than only leucine can be used as more effective dietary intervention (Wu, 2009). Activation of mTOR signaling by amino acid plays a central role on growth signal in placenta (Wen *et al.*, 2005) and protein synthesis in mammary gland (Bionaz *et al.*, 2011), linking maternal nutrition and fetal growth (Roos *et al.*, 2009).

Activation of mTOR signaling by amino acid plays a central role on growth signal in placenta (Wen *et al.*, 2005) and protein synthesis in mammary gland (Bionaz *et al.*, 2011), linking maternal nutrition and fetal growth (Roos *et al.*, 2009). However, maternal LP diet has been reported to not only decrease circulating BCAA, but also reduce mTOR signaling in the whole body. In dams fed an LP diet during pregnancy, placental amino acid transport systems were reduced via reduced mTOR signaling and signal transducer and activator of transcription 3 signaling (Rosario *et al.*, 2011). In addition, decreased activity of mTOR pathway mediated decrease of pancreatic cell proliferation in newborn and adult offspring (Alejandro.

E. U *et al.*, 2014). Thus, as activator of mTOR signaling, BCAA supplementation can potential solution for restricted protein environment. However, effects of maternal BCAA supplementation on growth and long-term health of offspring are poorly understood. Only few studies with fetus or neonates have reported protective effects of BCAA against intrauterine growth retardation caused by protein or food restriction (Teodoro *et al.*, 2012; Zheng C. A., 2009).

1.5. Aims of this study

The present study investigated the effects of maternal BCAA supplementation on protein metabolism of dams fed an LP diet and liver growth of their weaned offspring at postpartum day 21. In addition, dams and offspring were additionally analyzed at PP2 or 3 to determine whether maternal LP model was properly induced and maintained during pregnancy.

2. Materials and Methods

2.1. Animals and diets

Five-week-old virgin female CD-1 mice were obtained from Orient Bio Co. (Korea) and were maintained in a temperature (23 ± 2°C) and humidity (55 ± 5%)-controlled room with a 12 h dark/light cycle. After a one-week acclimation period with a chow diet, mice were randomly assigned to one of four groups. The NP group was fed a normal protein diet (20% casein), the LP group was fed a low protein diet (10% casein), the BCAA group was fed an LP diet with 2.1% BCAA, and the Ala group was fed an LP diet with 2.1% alanine. Ratio of isoleucine : leucine : valine in supplemented BCAA was 1 : 1.6 : 1.2, which is equivalent to the ratio of BCAA contained in casein. Experimental diets were prepared based on the AIN-93G diet formula (**Table 1**) and were provided *ad libitum* for 2 weeks before mating and throughout pregnancy and lactation (**Figure 1**). Dams were sacrificed and their serum or liver tissue were collected at two time point, at postpartum day (PP) 3 (n = 3) and PP 21 (n = 4), to determine the degree of catabolic state according to the LP diet intake period. Male offspring were sacrificed at postnatal day (PD) 2 to evaluate body and organ weights, and at PD 21 to determine postnatal liver development. All animal were fasted for 14 hour, weighed and anesthetized with intraperitoneal injections of 30 mg/kg Zoletil (Zoletil ® 50, Virbac, France) and 10 mg/kg xylazine

(Rompun®, Bayer Korea.co., Korea) before sacrifice. Serum and tissues were stored at –80°C for further analysis. All experimental procedures were approved by Seoul National University Institutional Animal Care and Use Committee (SNU-151019-6).

2.2. Serum and hepatic biochemical analyses

Serum glucose, triglyceride, total cholesterol and HDL-cholesterol, albumin, and blood urea nitrogen (BUN) levels were determined using commercial kits (Asan Pharmaceutical Co., Korea). Total protein levels were measured using a Bradford protein assay kit (Bio-Rad, USA). Hepatic DNA was extracted using a DNeasy blood & tissue kit (Qiagen, USA).

2.3. Serum free amino acid analyses

Serum free amino acid levels were determined by HPLC as previously described (Yoon *et al.*, 2017). Briefly, serum samples were mixed with 1 mmol/L norvaline as an internal standard and 20% sulphosalicylic acid to precipitate protein and kept for 60 min on ice. After the centrifugation at 12,000 × g for 5 min at 4°C, filtered samples were analyzed by the Ultimate 3000 HPLC system (Donex, Germany). The fluorescence intensities were observed at an excitation wavelength of 340nm and emission wavelength of 450 nm.

Table 1. Composition of experimental diet of dams

Ingredients (g/kg diet)	Diet			
	NP	LP	BCAA	Ala
Casein[1]	200.0	100.0	100.0	100.0
L-Isoleucine	-	-	5.4	-
L-Leucine	-	-	8.7	-
L-Valine	-	-	6.5	-
L-Alanine	-	-	-	20.6
L-Cystine	3.0	1.5	1.5	1.5
Corn Starch	397.5	499.0	478.4	478.4
Maltodextrin	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0
Soybean Oil	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0
Mineral Mix[2]	35.0	35.0	35.0	35.0
$CaHPO_4$	-	4.4	4.4	4.4
$CaCO_3$	2.539	-	-	-
Vitamin Mix[3]	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Total	1002.5	1004.4	1004.4	1004.4

[1]CA160030 (Harlan, USA)

[2]AIN-93G-MX (TD94046: Harlan, USA)

[3]AIN-93G-VX (TD94047: Harlan, USA)

NP, normal protein; LP, low protein; BCAA, low protein supplemented with BCAA (21 g/kg diet); Ala, low protein supplemented with alanine (21 g/kg diet)

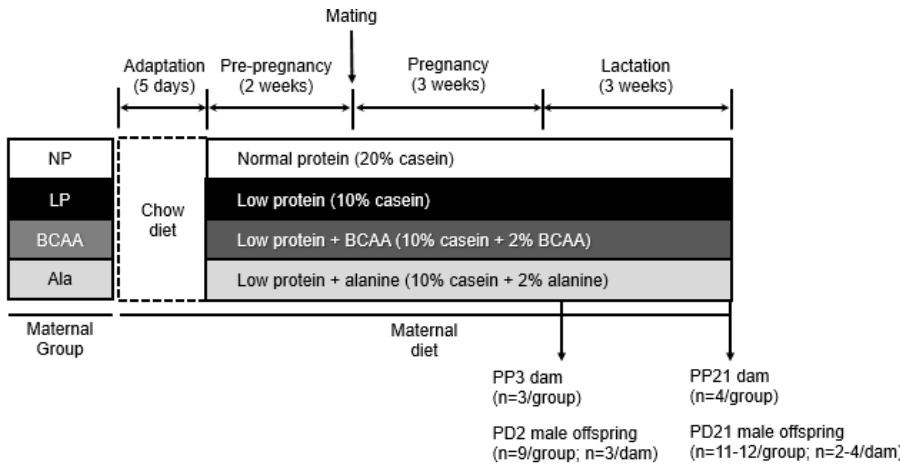

Figure 1. Diagram of experimental timeline.

2.4. Total protein extraction and immunoblotting

Approximately 25 mg of liver tissues were homogenized in 20 volumes (w:v) of ice-cold protein lysis buffer [50mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 0.1% Tween-20, 10% glycerol, Protease inhibitor cocktail (Sigma, USA)] using the TissueLyser II system (Qiagen, USA) with 5 mm sterile stainless steel beads. Equal amounts of protein were loaded into the lanes of a SDS-PAGE gel, separated, and blotted onto a PVDF membrane. After blocking with 5% nonfat milk or bovine serum albumin in TTBS, membranes were probed with a specific primary antibody, including proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, USA), cleaved form of caspase-3 (Cell Signaling Technology, USA), phospho-p70S6 kinase (p70S6k, Thr389; Cell Signaling Technology, USA), p70S6k (Cell Signaling Technology, USA), phospho-S6 ribosomal protein (p-S6, Ser235/236; Cell Signaling Technology, USA), S6 (Cell Signaling Technology, USA), or anti-70-kDa heat shock cognate protein (HSC70; Santa Cruz Biotechnology, USA). The membranes were then incubated with an IgG-peroxidase-conjugated secondary antibody with 5% nonfat milk or bovine serum albumin in TTBS for chemiluminescent detection. The band intensities were quantified using Quantity One software (Bio-Rad, USA).

2.5. Total RNA extraction and quantitative real-time PCR

Total RNA of liver tissue was isolated using RNAiso Plus (Takara, Japan). Approximately 25 mg of liver tissue was homogenized with 500 μL of RNAiso Plus and 5 mm sterile stainless steel beads using a TissueLyser II system (Qiagen, USA). The homogenate was left in ice for 15 minutes and 100 μL of chloroform was added and vigorously mixed for 15 seconds. Mixture was left in ice for 15 minutes and centrifuged at 12,000 x g for 15 min at 4 °C. Isopropanol was added to the supernatant, left in -20 °C for 1 hour, and centrifuged at 12,000 x g for 15 min at 4°C. After discarding the supernatant and rinsing with 500 μl of 75% ethanol, the RNA pellet was centrifuged at 7,500 x g for 2 min at 4 °C. The purified RNA precipitate was resuspended in diethylpyrocarbonate-treated water (DEPC-water), and concentration and purity of total RNA were measured by optical density at 260 and 280 nm. cDNA was synthesized using 2 μg of total RNA with the Superscript®II Reverse Transcriptase (Invitrogen, USA). All amplification reactions were performed using a StepOne™ Real Time PCR System (Applied Biosystems, USA) according to the manufacturer's protocol. The PCR condition was 40 cycles of the following protocol; denaturation at 95 °C for 10 seconds; and then annealing and extension at 60 °C for 1 minutes. Mouse β-actin (Acbt) was used as a reference gene and relative gene expression levels were analyzed using the $2^{-\Delta\Delta}$Ct method. Primer sequence is shown in **Table 2**.

Table 2. Quantitative real-time PCR primer sequences

Gene	Forward (5′-3′)	Reverse (5′-3′)
COL1A1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
HGF	ATGTGGGGGACCAAACTTCTG	GGATGGCGACATGAAGCAG
HNF4α	GCCAACGATCACCAAGCAAG	TGAGGGTATGAGCCAGCAGAA
LDLR	TGGCCATCTATGAGGACAAA	GTGTGACCTTGTGGAACAGG
SR-B1	CCTTCAATGACAACGACACCG	CCATGCGACTTGTCAGGCT
STAB2	CACTATGTCGGGGATGGACG	GGGAGCGTAGGTGGAATACG
α-SMA	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA
VEGF-A	CAGGCTGCTGTAACGATGAA	CTATGTGCTGGCTTTGGTGA
ACTB	GCTGAGAGGGAAATCGT	CGTCAGGCAGCTCATAG

COL1A1: collagen type 1 alpha 1, HGF: hepatocyte growth factor, HNF4α: hepatocyte nuclear factor 4 alpha, LDLR: low-density lipoprotein receptor, SR-B1: scavenger receptor class B1, STAB2: stabilin-2, α-SMA: alpha-smooth muscle actin, VEGF-A: vascular endothelial growth factor A, ACTB: β-actin

2.6. Hematoxylin and Eosin staining

Formalin-fixed liver tissues were cut into 4 μm sections. After removing the paraffin with xylene, tissue sections were stained with hematoxylin and eosin (H & E) for histological evaluation of the liver. Relative areas of hepatic sinusoid were quantified using ImageJ software (NIH, Bethesda, USA).

2.7. Statistical analysis

All results were analyzed by the One-way ANOVA followed by Duncan's multiple comparison test using SPSS software (version 22.0; IBM, USA). The differences were considered statistically significant at $P < 0.05$, and data were expressed as mean ± SEM. Correlation between two variables were analyzed by Pearson's correlation coefficient.

3. Results

3.1. Effects of BCAA supplementation on body and organ weights of dams fed an LP diet

To determine the effects of BCAA supplementation from pre-pregnancy to lactation on weights of dams, body and liver, kidney, retroperitoneal fat weights were measured in PP21 dams. In order to confirm the similarity with previous LP studies, the data of PP3 dams was represented first. Experimental diets for two weeks before mating did not affect body weight of females (NP, 28.22 ± 0.46 g; LP, 29.14 ± 0.66 g; BCAA, 29.91 ± 0.63 g; Ala, 29.03 ± 0.84 g). Body weight were not significantly different between NP and LP group in PP3 dams (**Table 3**) and these results are consistent with previous study (Cherala *et al.*, 2006; Zambrano *et al.*, 2005). However, there were no effects by maternal BCAA supplementation. Liver, kidney and retroperitoneal fat weights were also not significantly different among the groups. In PP21 dams, significant differences were not observed in body weight, liver and kidney weights among the groups (**Table 4**). However, absolute and relative weight of retroperitoneal fat were significantly increased in BCAA and Ala group compared with LP group. There were no significant differences in total liver DNA content among the groups both in PP3 and PP21 dams, suggesting maternal normal liver enlargement did not affected by maternal LP or BCAA diet.

Table 3. Body and organ weights of PP3 dams

	NP	LP	BCAA	Ala
Final body weight (g)	33.39 ± 1.63	31.30 ± 0.79	32.02 ± 1.06	32.48 ± 0.72
Absolute organ weight (g)				
Liver	1.65 ± 0.10	1.60 ± 0.08	1.49 ± 0.05	1.56 ± 0.04
Kidneys	0.37 ± 0.02	0.34 ± 0.02	0.37 ± 0.01	0.41 ± 0.02
Retroperitoneal fat	0.09 ± 0.03	0.16 ± 0.02	0.16 ± 0.04	0.14 ± 0.04
Relative organ weight (% BW)				
Liver	4.93 ± 0.13	5.10 ± 0.12	4.65 ± 0.18	4.80 ± 0.09
Kidneys	1.11 ± 0.02	1.08 ± 0.07	1.16 ± 0.03	1.28 ± 0.07
Retroperitoneal fat	0.26 ± 0.10	0.51 ± 0.04	0.51 ± 0.09	0.41 ± 0.13
Total liver DNA (mg)	7.0 ± 0.4	8.6 ± 1.2	7.9 ± 0.9	7.2 ± 1.1

Data are mean ± SEM (n = 3). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

Table 4. Body and organ weights of PP21 dams

	NP	LP	BCAA	Ala
Final body weight (g)	31.93 ± 1.80	29.33 ± 0.49	29.14 ± 0.73	28.60 ± 0.59
Absolute organ weight (g)				
Liver	1.71 ± 0.09	1.89 ± 0.10	1.49 ± 0.08	1.58 ± 0.19
Kidneys	0.46 ± 0.02	0.41 ± 0.03	0.40 ± 0.02	0.43 ± 0.04
Retroperitoneal fat	0.05 ± 0.01^{c}	0.06 ± 0.02^{bc}	0.16 ± 0.03^{a}	0.12 ± 0.02^{ab}
Relative organ weight (% BW)				
Liver	5.34 ± 0.04	6.45 ± 0.42	5.13 ± 0.22	5.51 ± 0.58
Kidneys	1.43 ± 0.06	1.41 ± 0.11	1.35 ± 0.05	1.52 ± 0.22
Retroperitoneal fat	0.14 ± 0.04^{c}	0.20 ± 0.06^{bc}	0.55 ± 0.09^{a}	0.42 ± 0.09^{ab}
Total liver DNA (mg)	6.7 ± 0.6	9.6 ± 1.6	6.5 ± 0.4	7.5 ± 1.0

Data are mean ± SEM (n = 4). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

3.2. Effects of BCAA supplementation on serum biochemical parameters of dams fed an LP diet

Although serum glucose, TG and TC levels of dams consumed LP diet have been reported to differ among the studies (Fernandez-Twinn *et al.*, 2003; Torres *et al.*, 2010), these indicators measured in PP3 and PP21 dams to determine the effect of BCAA supplementation on serum parameters of glucose and lipid metabolism. There were no significant differences in serum glucose, TG and TC levels among the groups in PP3 dams (**Table 5**). Also, no significant differences were observed in serum glucose, TG and TC levels among the groups in PP21 dams (**Table 6**). These finding indicates that maternal BCAA supplementation or LP diet had no effects on glucose and lipid metabolism of dams fed an experimental diet during pre-pregnancy and pregnancy and/or lactation in this study. However, interestingly, significantly higher serum leptin levels as compared to LP group were observed in BCAA and Ala group of PP21 dams. Serum leptin had significant correlation with retroperitoneal fat weights of PP21 dams ($r = 0.824$, $P < 0.001$). Since leptin levels adjusted to fat mass were not significant different among the groups, it seems that serum leptin levels were altered dependent with fat mass in PP21 dams.

Table 5. Serum and hepatic biochemical parameters of PP3 dams

	NP	LP	BCAA	Ala
Glucose (mg/dL)	138.0 ± 22.9	109.4 ± 16.9	132.1 ± 18.4	144.4 ± 20.1
TG (mg/dL)	27.1 ± 2.9	21.4 ± 4.8	44.3 ± 17.9	36.9 ± 15.4
TC (mg/dL)	132.5 ± 6.0	130.2 ± 12.6	128.6 ± 4.5	140.6 ± 12.2

Data are mean ± SEM (n = 3). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

Table 6. Serum and hepatic biochemical parameters of PP21 dams

	NP	LP	BCAA	Ala
Glucose (mg/dL)	174.9 ± 18.3^{a}	64.7 ± 9.6^{b}	77.7 ± 12.8^{b}	76.1 ± 11.4^{b}
TG (mg/dL)	41.9 ± 5.1	35.9 ± 6.2	46.8 ± 10.7	38.7 ± 8.3
TC (mg/dL)	204.8 ± 8.8	151.5 ± 46.5	174.7 ± 24.2	155.9 ± 24.9
Leptin (ng/mL)	0.9 ± 0.3^{b}	0.9 ± 0.2^{b}	4.4 ± 1.2^{a}	3.2 ± 0.9^{ab}

Data are mean ± SEM (n = 4). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

3.3. Effects of BCAA supplementation on serum free amino acid of dams fed an LP diet

As good metabolic indicators of dams and prime substrate of offspring growth, serum free amino acid levels were measured in PP3 and PP21 dams. Especially, this measurement was focused on serum BCAA levels, as an amino acid which is initially reduced in dams fed an LP diet during pregnancy (Kwong *et al.*, 2000; Parimi *et al.*, 2004) and potential mediator between protein status of dams and growth of offspring. Unlike expectation, maternal LP diet before mating and during pregnancy did not decrease serum BCAA levels as compared to NP group in PP3 dams (**Table 7**). Maternal BCAA supplementation did not affect serum BCAA levels compared with LP group in PP3 dams. In PP21 dams, serum BCAA levels were also not significantly different between NP and LP group (**Table 8**). There were also no significant differences between BCAA and LP group in PP21 dams. Interestingly, most amino acids including asparagine, glutamine, glycine, lysine, proline, serine and threonine levels were significantly increased in LP group compared with NP group at PP21. Among these amino acids which were significantly higher in LP group, threonine was significantly reduced only in BCAA group, and glutamine and glycine were significantly decreased both in BCAA and Ala group than in LP group. Tryptophan was the only amino acid that was significantly lower in LP group than in NP group, and was tended to be higher in BCAA group and was significantly increased in Ala group.

Table 7. Serum free amino acid profiles of PP3 dams

(μmol/L)	NP	LP	BCAA	Ala
Alanine	527.1 ± 48.0	639.6 ± 167.5	637.1 ± 176.1	547.7 ± 7.4
Arginine	121.4 ± 7.2	150.0 ± 13.4	142.5 ± 8.4	138.4 ± 9.7
Asparagine	50.0 ± 2.6	61.7 ± 6.3	51.8 ± 12.3	47.0 ± 1.9
Aspartate	9.4 ± 1.9	10.4 ± 0.8	66.6 ± 1.9	59.6 ± 1.5
Glutamine	424.9 ± 38.2	447.2 ± 63.9	397.2 ± 45.9	400.7 ± 15.7
Glutamate	73.4 ± 4.7	51.9 ± 9.6	69.2 ± 6.4	60.8 ± 8.7
Glycine	182.8 ± 3.7	262.3 ± 22.6	228.4 ± 55.2	229.8 ± 3.2
Histidine	76.9 ± 3.0	91.5 ± 10.6	103.5 ± 22.0	83.5 ± 3.2
Isoleucine	172.5 ± 24.1	149.9 ± 26.1	107.3 ± 2.0	101.9 ± 13.6
Leucine	107.3 ± 12.3	103.0 ± 17.9	69.3 ± 2.0	73.8 ± 8.7
Lysine	592.0 ± 85.5	739.4 ± 79.2	651.3 ± 51.5	587.7 ± 49.1
Methionine	58.7 ± 2.0^{a}	49.3 ± 1.8^{ab}	44.6 ± 5.6^{b}	41.5 ± 2.1^{b}
Phenylalanine	75.7 ± 4.8^{a}	56.8 ± 3.8^{b}	53.2 ± 6.3^{b}	49.3 ± 3.9^{b}
Proline	83.4 ± 6.9	82.1 ± 5.6	76.2 ± 11.4	75.3 ± 3.5
Serine	206.6 ± 10.2	397.1 ± 73.6	339.8 ± 86.0	333.3 ± 15.6
Threonine	322.9 ± 22.1	349.0 ± 6.8	291.5 ± 7.2	306.9 ± 31.5
Tryptophan	59.5 ± 2.6	51.5 ± 4.5	51.7 ± 7.9	49.2 ± 7.6
Tyrosine	87.0 ± 3.0^{a}	54.9 ± 6.6^{b}	57.1 ± 8.5^{b}	43.9 ± 5.2^{b}
Valine	232.1 ± 24.8^{a}	183.7 ± 2.1^{ab}	149.8 ± 2.1^{b}	142.8 ± 16.4^{b}
BCAA	512.0 ± 61.3	436.59 ± 63.5	326.5 ± 1.9	318.5 ± 38.3
EAA	1697.7 ± 162.5	1774.0 ± 154.3	1522.2 ± 81.3	1438.6 ± 116.6
NEAA	1766.2 ± 90.4	2157.3 ± 339.5	2008.3 ± 395.7	1884.4 ± 41.0

Data are mean ± SEM (n = 3). Means in the same row that do not share the same alphabetic superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

Table 8. Serum free amino acid profiles of PP21 dams

(μmol/L)	NP	LP	BCAA	Ala
Alanine	168.9 ± 30.3	438.8 ± 125.2	291.5 ± 47.3	383.6 ± 33.5
Arginine	80.0 ± 7.5	99.6 ± 17.1	137.8 ± 6.6	110.2 ± 30.8
Asparagine	33.2 ± 2.7^{b}	54.4 ± 5.4^{a}	45.9 ± 4.7^{ab}	54.5 ± 1.5^{a}
Aspartate	5.2 ± 0.2^{b}	8.4 ± 1.6^{ab}	8.0 ± 0.8^{ab}	10.7 ± 0.7^{a}
Glutamine	300.3 ± 27.8^{b}	516.3 ± 54.3^{a}	351.8 ± 15.8^{b}	375.4 ± 22.0^{b}
Glutamate	29.1 ± 3.0	54.7 ± 12.8	54.8 ± 1.1	58.4 ± 6.0
Glycine	86.4 ± 1.7^{c}	274.9 ± 40.3^{a}	158.9 ± 28.0^{bc}	186.2 ± 8.1^{b}
Histidine	50.3 ± 2.8	328.1 ± 127.2	109.1 ± 14.8	123.4 ± 8.3
Isoleucine	110.0 ± 24.6	77.8 ± 7.7	85.2 ± 3.3	72.5 ± 6.5
Leucine	182.9 ± 46.8	141.9 ± 14.2	125.0 ± 8.1	111.5 ± 3.6
Lysine	119.4 ± 26.8^{b}	627.8 ± 120.9^{a}	418.0 ± 69.2^{a}	574.8 ± 31.4^{a}
Methionine	38.5 ± 1.2	41.4 ± 1.1	40.0 ± 4.1	45.2 ± 1.0
Phenylalanine	61.9 ± 4.3	68.6 ± 10.3	57.9 ± 4.9	61.7 ± 9.1
Proline	39.0 ± 1.9^{b}	67.4 ± 3.1^{a}	61.7 ± 6.0^{a}	73.4 ± 8.3^{a}
Serine	102.5 ± 11.6^{b}	298.2 ± 45.4^{a}	241.7 ± 59.4^{a}	271.0 ± 21.6^{a}
Threonine	137.6 ± 12.1^{b}	450.7 ± 103.7^{a}	230.4 ± 33.6^{b}	301.0 ± 17.9^{ab}
Tryptophan	62.0 ± 5.4^{a}	29.7 ± 4.8^{c}	39.9 ± 5.9^{bc}	47.2 ± 4.6^{ab}
Tyrosine	37.5 ± 2.3	65.5 ± 11.1	70.9 ± 14.5	65.9 ± 1.1
Valine	235.0 ± 35.0	253.9 ± 55.9	186.8 ±14.9	186.7 ± 1.1
BCAA	527.8 ± 106.3	473.7 ± 65.7	397.0 ± 25.1	370.7 ± 8.9
EAA	997.5 ± 143.3	2019.9 ± 418.1	1292.2 ± 149.2	1523.9 ± 8.3
NEAA	882.2 ± 55.9^{b}	1878.3 ± 268.6^{a}	1423.1 ± 140.0^{a}	1589.3 ± 66.7^{a}

Data are mean ± SEM (n = 4). Means in the same row that do not share the same alphabetic superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PP, Postpartum day; NP, Normal protein diet group; LP, Low-protein diet group; BCAA, Low-protein with BCAA diet group; Ala, Low-protein with Ala diet group

3.4. Effects of BCAA supplementation on hepatic protein synthesis and degradation of dams fed an LP diet

To determine the effects of maternal BCAA supplementation on protein metabolism of dams fed an LP diet, protein metabolism-related indicators were measured in PP3 and PP21 dam. Additionally, activation of mTOR signaling by BCAA supplementation was determined by protein levels of phosphorylated or total form of mTOR, p70S6k or ribosomal protein S6. No significant differences were observed in serum total protein, albumin, BUN and hepatic total protein levels among the groups in PP3 dams (**Figure 2A**). Protein levels of p-p70S6, p70S6, p-S6 and total S6 were also not significantly different among the groups in PP3 dams (**Figure 2B**). There were no significant differences in protein degradation determined by ubiquitinated protein in PP3 dams (**Figure 2C**). In contrast, serum albumin, hepatic total protein and hepatic total S6 protein levels, which were significantly lower in LP group than in NP group, were significantly increased in BCAA and Ala group compared with LP group (**Figure 3A**). p-S6 levels which were significantly lower in LP group than in NP group were not affected by maternal BCAA supplementation (**Figure 3B**). As PP3 dams, PP21 dams had no significant differences in expression pattern of ubiquitinated protein (**Figure 3C**), suggesting that alteration of protein levels observed in PP21 dams was resulted from protein synthesis, not from protein degradation.

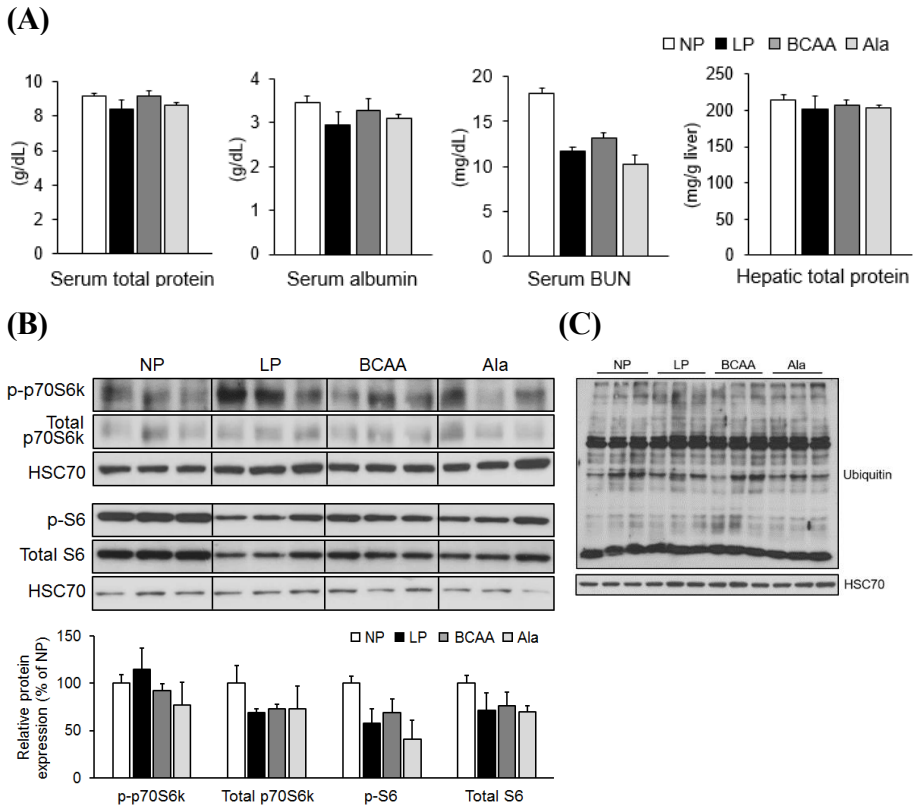

Figure 2. Effects of BCAA supplementation on hepatic protein synthesis and degradation in dams fed an LP diet at PP3.

(A) Serum total protein, albumin BUN and hepatic total protein levels in PP3 dams (n = 3). (B) Relative protein levels of hepatic p-p70S6k, p70S6k, p-S6, S6 in PP3 dams. HSC70 was used as an endogenous control to normalize the band. Data are expressed as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$). (C) Ubiquitinated protein in PP3 dams (n = 3).

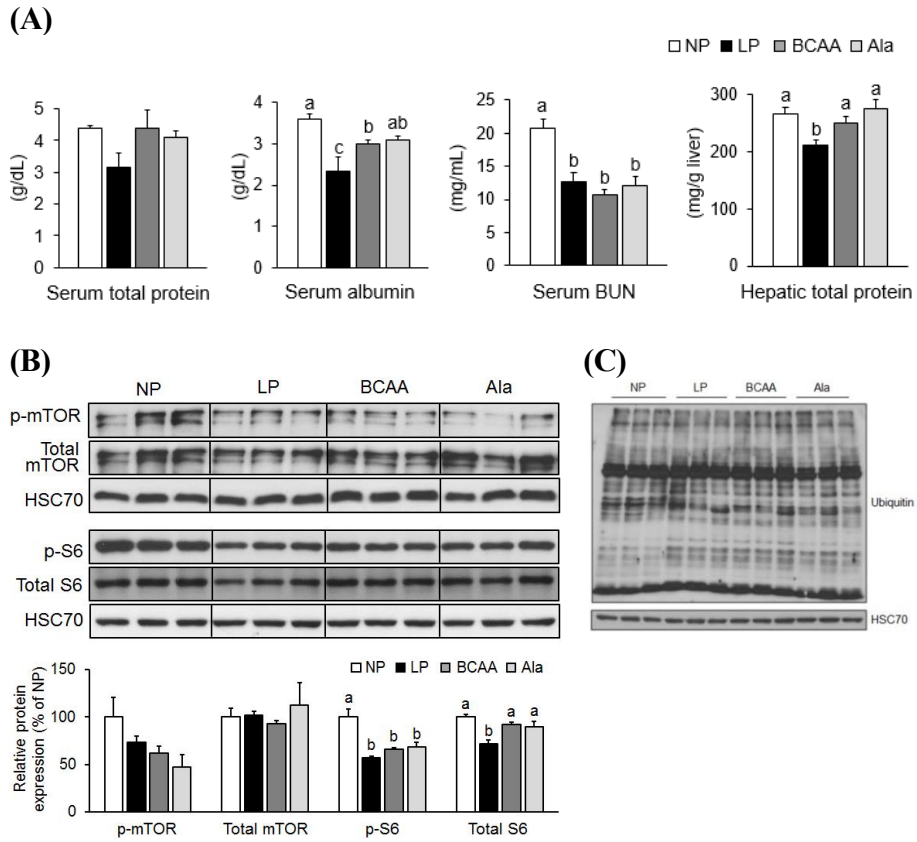

Figure 3. Effects of BCAA supplementation on hepatic protein synthesis and degradation in dams fed an LP diet at PP21.

(A) Serum total protein, albumin BUN and hepatic total protein levels in PP21 dams (n = 4) (B) Relative protein levels of hepatic p-p70S6k, p70S6k, p-S6, S6 in PP21 dams. HSC70 was used as an endogenous control to normalize the band. Data are expressed as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$). (C) Ubiquitinated protein in PP3 dams (n = 3).

3.5. Effects of maternal BCAA supplementation on body weight and organ weights of offspring from dams fed an LP diet

To determine the effects of maternal LP diet on fetal growth, body weight and organ weights were measured in new-born mice at postnatal day 2. After then, body weight was measured once a week to evaluate growth, and organ weights were measured at sacrifice on the postnatal day 21. Maternal LP diet significantly decreased body weight of offspring at PD2, which were not affected by maternal BCAA supplementation (**Table 9**). Liver and kidneys weights were not significantly different among the groups in PD2 offspring. In contrast, during weaning period, body weights of offspring were consistently lower in LP-O group than those of NP-O group (**Table 10**). Supplementation of Ala, but not BCAA, on maternal LP diet significantly increased body weight of offspring at PD20. In PD21 offspring, absolute weight of liver, kidneys, epididymal fat, brain and spleen were significantly lower in LP-O group than in NP-O group (**Table 11**). Maternal LP diet significantly reduced relative epididymal fat and spleen weights, and increased relative brain weight in LP-O group as compared to NP-O group. These results about absolute and relative organ weights were consistent with previous studies (Desai *et al.*, 1996; Maloney *et al.*, 2003). Maternal BCAA supplementation did not significantly affect absolute and relative organ weights of LP-O group in PD21 offspring.

Table 9. Body weight and organ weights of PD2 offspring

	NP-O	LP-O	BCAA-O	Ala-O
Body weight at sacrifice (g)	2.10 ± 0.04[a]	1.90 ± 0.07[b]	1.83 ± 0.09[b]	1.73 ± 0.04[b]
Absolute organ weight (mg)				
Liver	74.00 ± 3.84	66.78 ± 4.39	68.89 ± 3.83	61.78 ± 3.92
Kidneys	20.11 ± 1.57	19.22 ± 1.40	21.44 ± 2.70	19.67 ± 1.22
Relative organ weight (%BW)				
Liver	3.53 ± 0.20	3.53 ± 0.18	3.77 ± 0.11	3.54 ± 0.16
Kidneys	0.70 ± 0.18	1.01 ± 0.06	1.16 ± 0.07	1.14 ± 0.08

Data are mean ± SEM (n = 9). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

Table 10. Body weight changes of offspring

	NP-O	LP-O	BCAA-O	Ala-O
PD3	2.85 ± 0.11^{a}	1.94 ± 0.05^{b}	1.91 ± 0.05^{b}	2.12 ± 0.06^{b}
PD7	5.52 ± 0.19^{a}	3.46 ± 0.08^{b}	3.26 ± 0.11^{b}	3.54 ± 0.08^{b}
PD14	9.59 ± 0.17^{a}	5.05 ± 0.13^{b}	5.20 ± 0.11^{b}	5.49 ± 0.17^{b}
PD20	14.05 ± 0.32^{a}	6.94 ± 0.35^{c}	7.10 ± 0.22^{bc}	7.86 ± 0.19^{b}

Data are mean ± SEM (n = 11-12). Means in the same row that do not share the same alphabetic superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

Table 11. Body weight and organ weights of PD21 offspring

	NP-O	LP-O	BCAA-O	Ala-O
Final body weight at sacrifice (g)	12.48 ± 0.28^{a}	6.25 ± 0.31^{c}	6.39 ± 0.20^{bc}	7.01 ± 0.16^{b}
Absolute organ weight (g)				
Liver	0.51 ± 0.01^{a}	0.25 ± 0.01^{b}	0.25 ± 0.01^{b}	0.27 ± 0.01^{b}
Kidneys	0.18 ± 0.00^{a}	0.08 ± 0.00^{c}	0.08 ± 0.00^{c}	0.10 ± 0.00^{b}
Epididymal fat	0.10 ± 0.01^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}
Brain	0.44 ± 0.01^{a}	0.39 ± 0.01^{b}	0.39 ± 0.00^{b}	0.39 ± 0.00^{b}
Spleen	0.12 ± 0.01^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.04 ± 0.00^{b}
Relative organ weight (%BW)				
Liver	4.08 ± 0.06^{a}	3.94 ± 0.04^{ab}	3.86 ± 0.05^{b}	3.91 ± 0.06^{b}
Kidneys	1.42 ± 0.02^{a}	1.30 ± 0.06^{ab}	1.30 ± 0.02^{b}	1.40 ± 0.03^{ab}
Epididymal fat	0.83 ± 0.08^{a}	0.28 ± 0.05^{b}	0.25 ± 0.04^{b}	0.29 ± 0.04^{b}
Brain	3.57 ± 0.07^{c}	6.31 ± 0.26^{a}	6.12 ± 0.19^{ab}	5.66 ± 0.13^{b}
Spleen	0.94 ± 0.07^{a}	0.48 ± 0.02^{c}	0.52 ± 0.05^{bc}	0.62 ± 0.02^{b}

Data are mean ± SEM (n = 11-12). Means in the same row that do not share the same superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

3.6. Effects of maternal BCAA supplementation on serum and hepatic biochemical parameters in offspring from dams fed an LP diet

Maternal LP diet before mating and throughout pregnancy and lactation significantly decreased serum glucose, TG, HDL/TC ratio, total protein, albumin and hepatic total protein levels as compared to NP group, obviously representing the growth restriction (**Table 12**). Maternal BCAA supplementation did not significantly affect the levels of these biochemical parameters of LP-O group. Meanwhile, hepatic total protein levels were significantly increased in Ala-O group compared with LP-O group in PD21 offspring. Serum leptin levels were not significantly different between NP-O and LP-O group or between LP-O group and BCAA-O group, and had positive correlation with epididymal fat weight of PD21 offspring (r = 0.703, P = 0.002).

Table 12. Serum and hepatic biochemical parameters of PD21 offspring

	NP-O	LP-O	BCAA-O	Ala-O
Serum				
Glucose (mg/dL)	125.2 ± 9.0[a]	82.8 ± 6.7[b]	82.1 ± 9.8[b]	93.7 ± 8.4[b]
TG (mg/dL)	43.9 ± 5.6[a]	27.8 ± 2.6[b]	25.8 ± 1.3[b]	29.5 ± 1.4[b]
TC (mg/dL)	93.5 ± 4.4	100.0 ± 6.2	98.5 ± 3.4	91.2 ± 4.3
HDL-C (mg/dL)	40.6 ± 4.0	32.6 ± 3.5	30.2 ± 2.2	30.3 ± 2.5
HDL-C/TC ratio	2.5 ± 0.2[b]	3.4 ± 0.4[a]	3.4 ± 0.2[a]	3.2 ± 0.2[ab]
Total protein (g/dL)	3.8 ± 0.1[a]	3.3 ± 0.1[b]	3.4 ± 0.0[b]	3.3 ± 0.1[b]
Albumin (g/dL)	3.1 ± 0.1[a]	2.6 ± 0.1[b]	2.7 ± 0.0[b]	2.6 ± 0.0[b]
BUN (mg/dL)	22.8 ± 1.2	19.4 ± 1.9	22.7 ± 2.3	20.7 ± 0.8
Leptin (ng/mL)	1.8 ± 0.4[a]	1.2 ± 0.4[ab]	0.5 ± 0.1[b]	0.5 ± 0.1[b]
Liver				
Total protein (mg/g liver)	241.6 ± 7.4[a]	213.0 ± 5.4[b]	227.1 ± 8.7[ab]	237.0 ± 7.4[a]
DNA (mg/g liver)	1.6 ± 0.2	1.2 ± 0.5	1.6 ± 0.5	1.6 ± 0.2

Data are mean ± SEM (n = 11-12). Means in the same row that do not share the same alphabetic superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

3.7. Effects of maternal BCAA supplementation on hepatic proliferation and apoptosis in offspring from dams fed an LP diet

Although maternal LP diet has been reported to decrease proliferation and to increase apoptosis in fetal liver (Ramadan *et al.*, 2013), there were few reports that investigated the effects of maternal LP diet on postnatal liver development of weaned offspring. Thus, to determine the effects of maternal BCAA supplementation on proliferation and apoptosis in liver of young offspring, proliferating cell nuclear antigen (PCNA) and active form of caspase-3 protein levels were measured in liver of PD21 offspring. Hepatic PCNA levels was significantly higher in LP-O group compared with NP group (**Figure 4**). Postnatal mice liver represents high proliferation from 5-old-day to 15-old day as represented by increased PCNA and β-catenin, but after day 20, these proliferation are dramatically decreased and hepatocytes enter the stage of maturation (Reed *et al.*, 2008). Thus, higher PCNA levels in LP group than in NP group may reflect the retarded maturation of hepatocyte. However, maternal BCAA supplementation did not significantly affect the PCNA levels of LP-O group. β-catenin, a protein bound to intracellular cytoplasmic E-cadherin and an important factor of hepatocyte proliferation and postnatal liver development (Apte *et al.*, 2007), did not show significant differences among the groups. Although apoptosis based on caspase-3 cleavage, tended to be higher in LP group, differences did not reach to statistical significance.

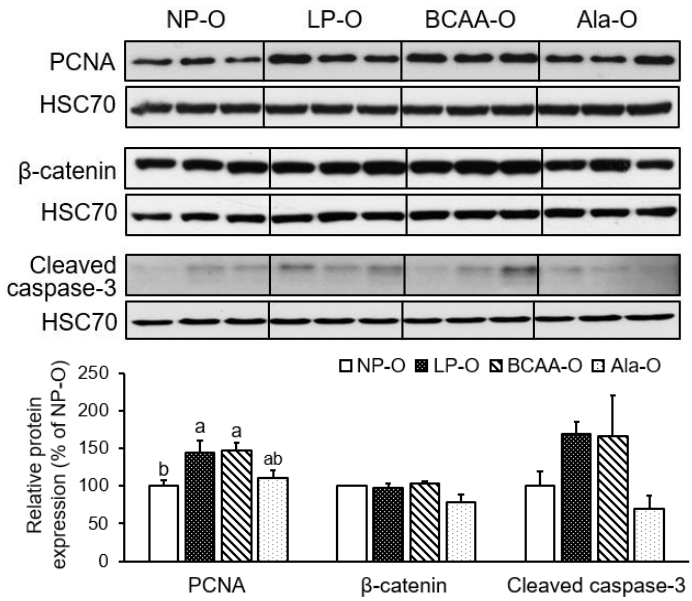

Figure 4. Effects of maternal BCAA supplementation on hepatic proliferation and apoptosis of PD21 offspring.

Relative protein levels of PCNA, β-catenin and cleaved caspase-3 in liver of PD21 offspring. HSC70 was used as an endogenous control to normalize the band. Data are expressed as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

PCNA; Proliferating cell nuclear antigen, NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

3.8. Effects of BCAA supplementation on liver development of offspring from dams fed an LP diet

In relation to increased hepatic proliferation determined to PCNA levels in LP-O group, liver sections were stained and observed to determine the effects of maternal BCAA supplementation on retardation of structural development of liver in PD21 offspring. Interestingly, apparent decrease of hepatic sinusoids, portal venules and central venules was observed in PD21 offspring, and these decrease of vessels seemed to have recovered in BCAA and Ala group compared with LP group (**Figure 5A**). Quantified area of liver sinusoids was also significantly lower in LP group than in NP group, but was significantly increased in BCAA and Ala group as compared to LP group (**Figure 5B**). Gene expression levels of HNF4α, a hepatic core growth factor which important to early- and postnatal hepatic vasculature development and maintenance (Yin. L *et al.*, 2011), were significantly lower in LP-O group (**Figure 5C**). Hepatocyte growth factor, a typical regulator of development of liver, and vascular endothelial growth factor A, a typical angiogenesis factor, showed similar pattern with HNF4α but the difference did not reach the statistical significance. Additionally, collagen type 1 alpha 1, alpha-smooth muscle actin and stabilin-2 were measured as a marker of endothelial cell and liver sinusoid, but were not significantly different among the groups in PD21 offspring. Since one of the most important roles of hepatic sinusoids is the clearance of various substances, particularly HDL-C and LDL-C (Landschulz *et al.*, 1996),

receptors which are located at the liver sinusoid, were measured additionally. LDLR mRNA levels was significantly lower in LP-O offspring, but maternal BCAA supplementation did not restore these decrease of LDLR levels in offspring as compared to LP-O offspring.

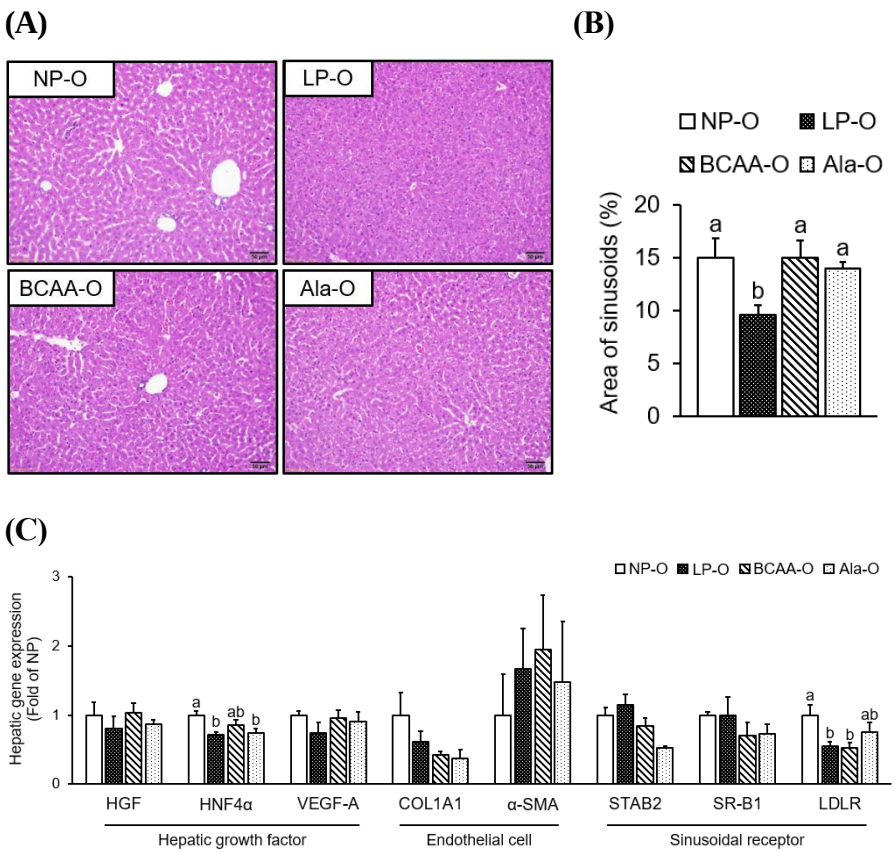

Figure 5. Effects of maternal BCAA supplementation on hepatic morphology, growth factor and sinusoidal receptors of PD21 offspring.

(A) H&E staining of liver section (original magnification x 200) of PD21 offspring. (B) Relative area of hepatic sinusoids of PD21 offspring was quantified by imageJ software. (C) Relative mRNA expression of hepatic growth factor and receptors which are located on liver sinusoid. Mouse ACBT was used as an internal control to normalize the data, and the fold of induction was calculated with the expression in the NP-O group. Data are expressed as mean ± SEM (n = 4). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

NP-O; Offspring of normal protein diet group, LP-O; Offspring of low-protein diet, BCAA-O; Offspring of low-protein with BCAA diet group, Ala-O; Offspring of low-protein with Ala diet group

4. Discussion

There is increasing epidemiological and experimental evidence representing that prenatal and postnatal nutrition affect metabolic phenotype in dams and offspring, and ultimately contribute to metabolic dysregulation in early and later life (Mcmillen *et al.*, 2005; Salam *et al.*, 2014). In this sense, the effects of BCAA supplementation on protein metabolism of dams fed an LP diet before mating and throughout pregnancy and lactation were investigated. These periods contain all time-windows that have been reported to program fetal development and adult metabolism (Symonds *et al.*, 2004). Additionally, the effects of maternal consumption of BCAA supplemented to LP diet on liver development of weaned offspring were observed.

In this study, serum BUN and albumin levels were significantly lower in LP dams at PP21. Low BUN levels represent reduced protein oxidation and protein turnover. Also, albumin is a protein that accounts for 10% of intrahepatic protein synthesis, performing a variety of biological functions including ligand binding, antioxidant, and anti-inflammation (Quinlan *et al.*, 2005). Hypoalbuminemia reflects a pathological condition including chronic liver disease (Quinlan *et al.*, 2005). Based on these facts, reduced BUN and albumin levels in LP groups of PP21 dams mean inhibited protein metabolism. Increase of serum albumin levels in BCAA and Ala group of PP21 dams indicates that maternal BCAA and Ala supplementation have

the same effects on restoration of hepatic albumin synthesis in dams fed an LP diet.

In addition, maternal BCAA or Ala supplementation significantly increased maternal leptin levels as compared to LP group of PP21 dams. It has been reported that leptin increases expression of placental system A (Von Versen-Hoynck *et al.*, 2009), and maternal LP diet during pregnancy decreases maternal leptin levels (Jansson *et al.*, 2006; Rosario *et al.*, 2011). Thus, leptin is an anabolic hormone which sensitively responds to maternal protein restriction and can determine transportation of amino acids to fetus. Because maternal serum leptin levels during pregnancy or activity of placental system A were not measured, this assumption cannot be verified in present study. Instead, there was positive correlation between serum leptin levels and hepatic total protein levels of PP21 dam ($r = 0.523$, $P = 0.038$). Also, maternal leptin secreted during lactation has been reported to regulate energy utilization in conjunction with other hormone including prolactin in dams (Mukherjea *et al.*, 1999). Thus, increased leptin levels in PP21 dams by supplementation of BCAA or Ala may have affected increased hepatic protein synthesis of dams. Indeed, maternal leptin levels were not significantly affected by LP diet. There are several possibilities why leptin levels of PP21 dams were increased only in BCAA and Ala groups. First, the difference in composition of experimental diet can be the cause of these finding. In PP21 dams, serum leptin levels seems to be changed dependent on maternal retroperitoneal fat mass. It is unclear why

fat weight was increased only in BCAA and Ala dams, but manner of dietary modification should be considered. Crystalline amino acids supplemented on maternal diet is quickly absorbed in the intestinal tract and can temporarily cause amino acid imbalance (Wu, 2009). In other words, BCAA or Ala supplemented on LP diet can induce not only a simple increase of nitrogen, but also a rapid change of maternal amino acid profile. However, amino acid supplementation within 2.5% of the diet does not cause toxicity and has greater benefits for offspring growth (Wu, 2009). Thus, BCAA or Ala supplemented as 2.1% on maternal LP diet may be rapidly metabolized and used in tissue of LP dams, which were on catabolic state and had increased demand of protein and amino acids. Second, BCAA, especially leucine, can regulate leptin secretion through activation of mTOR signaling in adipose tissue as well as skeletal muscle (Li *et al.*, 2011). Adipose tissue expresses the greatest amount of mTOR per milligram of tissue protein and gives the most strong response in protein synthesis to chronic leucine supplementation (Lynch *et al.*, 2002), presenting the possibility that adipose tissue sensitively responded to maternal amino acid supplementation. Activated mTOR signaling in adipose tissue can increase protein synthesis, leptin secretion, and lipogenesis through increase in Sterol regulatory element-binding protein 1/2 and Peroxisome proliferator-activated receptor-γ gene (Laplante *et al.*, 2012). Therefore, there are possibilities that mTOR signaling in maternal adipose tissue responded to supplementation of BCAA or Ala added as free amino acid on LP diet and

resulted in increase of lipogenesis and leptin secretion. Also, leptin itself can activate mTOR signaling in adipose tissue and hepatocyte (Rodriguez *et al.*, 2011). In this study, maternal serum leptin and hepatic total protein content had a positive correlation (r = 0.523, P = 0.038), supposing that high leptin levels in serum of BCAA or Ala dams contributed elevation of hepatic protein synthesis. The result that total S6 levels in liver of BCAA and Ala group of PP21 dams supports these presumption, which seems to be responsible for increase of serum albumin and hepatic total protein levels in dams. Although hepatic p-S6 levels had a positive correlation with serum albumin (r = 0.739, P = 0.006), there were no significant increase of p-S6 levels in BCAA and Ala group of PP21 dams. These results suggests that the hepatic protein synthesis in dams may be affected by other factors such as insulin, IGF-1 and amino acids. Overall, based on serum leptin and hepatic total S6 protein levels, maternal BCAA and Ala supplementation may have affected both liver and adipose tissue.

Circulating BCAA levels have been reported to be sensitively reduced in serum of dams fed an LP diet during early-gestation, whole gestation (Bhasin *et al.*, 2009; Eckert *et al.*, 2012). In addition, significantly lower BCAA levels as compared to control group were also observed in dams fed an LP diet during pregnancy and 1–2 weeks postpartum (Bhasin *et al.*, 2009). However, PP21 dams were different with the previous studies in two point. First, there was no significant difference in serum BCAA levels among the groups of PP21 dams. In early-pregnancy, serum BCAA levels

of dams fed a severe LP diet containing 6% protein during pregnancy were significantly decreased compared with control group, but this difference between the groups was diminished in late-pregnancy (Parimi *et al.*, 2004). Thus, BCAA level change seems to be sensitive to initial term of LP diet consumption. Second, most amino acids were tended to be higher or significantly increased in LP group of PP21 dams. This finding is discordant with previous studies that have been reported the reduction of serum EAA including BCAA (Bhasin *et al.*, 2009; Jansson *et al.*, 2006; Kwong *et al.*, 2000; Rees *et al.*, 1999), threonine (Kwong *et al.*, 2000; Wang *et al.*, 2015) and histidine (Kwong *et al.*, 2000; Wang *et al.*, 2015) in dams fed an LP diet during pregnancy. However, to our best knowledge, there is no study about amino acid profile of dams fed an LP diet from pre-pregnancy to end of lactation. There are several possible explanations for hyperaminoacidemia in PP21 dams fed an LP diet. First, the effects of LP diet on maternal BCAA or EAA levels seems to be altered by severity or period of protein restriction. Maternal severe LP diet initially decreases plasma BCAA levels of dams, but in late-pregnancy, LP diet consumption significantly increased both plasma EAA and NEAA levels of dams (Parimi *et al.*, 2004). In other words, the patterns of BCAA, EAA and NEAA levels of dams fed a severe LP diet (6% casein) during pregnancy (Parimi *et al.*, 2004) were similar with PP21 dams fed a moderate LP diet (10% casein) during pre-pregnancy, pregnancy and lactation. Because LP groups of PP3 dams did not show increase of amino acids compared with

NP dams, LP diet consumption for two weeks before mating may have offset the change of serum BCAA levels of dams in present study. Second, low uptake of amino acids into tissues may have affected high serum amino acid levels in LP dams. Maternal LP diet induces dams to prefer lipids as an energy source, and causes an adaptive response that decreases gluconeogenesis from available amino acids (Holness *et al.*, 1998). Small-for-gestational age infants who had undergone intrauterine malnutrition are shown to hyperglycemia and hyperaminoacidemia due to reduced hepatic gluconeogenic capacity and limited hepatic uptake of glucogenic amino acids (Mestyan *et al.*, 1975). In the same study, low plasma glucose levels and high amino acids levels in SGA infants had a negative correlation (Mestyan *et al.*, 1975). PP21 dams also had a negative correlation between serum glucose which were lower in LP dams and serum amino acids including asparagine, glutamate, glycine, lysine and proline which were higher in LP dams compared with NP dams. Interestingly, serum glucose had a positive correlation with leucine ($r = 0.701$, $P = 0.011$), isoleucine ($r = 0.711$, $P = 0.004$) and BCAA levels ($r = 0.594$, $P = 0.042$) in PP21 dams. Oxidized BCAA can provide nitrogen for the synthesis and release of glucogenic amino acids, especially glutamine and alanine (Baracos *et al.*, 2006). In calorie-restricted dams, maternal BCAA supplementation significantly increased protein levels of fructose-1, 6-biphosphatase and phosphoenolpyruvate carboxykinase as well as IGF-Ⅰ in fetal liver (Cao *et al.*, 2009). Based on these results and researches, positive correlation

between serum glucose and BCAA of PP21 dams may be resulted from the gluconeogenic capacity of BCAA. Since amino acids levels within maternal tissue and activity of gluconeogenic enzymes of dams were not measured in this study, further study is needed about the effects of maternal LP diet or BCAA supplementation on glucogenic amino acid and gluconeogenesis of dams. Another possibility is that the plasma volume is reduced in LP dams, and the concentration of serum amino acids are increased. In PP21 dams, we observed a lower albumin content in the serum from LP dams. As albumin is the main factor maintaining colloid osmotic pressure in plasma and increase the plasma volume (Quinlan *et al.*, 2005), decrease of albumin levels may have decreased the plasma volume. Also, protein-energy malnutrition during pregnancy has been reported to reduce maternal plasma volume, (Rosso *et al.*, 1979) and it was considered that the decrease of plasma protein, especially albumin, may affect the decrease of plasma volume in protein-energy deficient dams. Therefore, hypoalbuminemia observed in LP dams seems to contribute a lower plasma volume, and abnormal increase of most amino acid concentration.

In this study, weaned offspring were analyzed to investigate whether maternal BCAA supplementation could restore growth restriction and retardation of liver development of offspring from dams fed an LP diet. LP offspring showed growth restriction and catabolic states including lower body weight, organ weight and serum TG, total protein, albumin, HDL/TC ratio as well as LP dams. Supplementation of Ala, but not BCAA, on

maternal LP diet significantly increased body weight of offspring at PD20 and PD21. Maternal consumption of LP diet significantly decreased protein levels in serum and liver of offspring. Although BCAA and alanine supplementation stimulated protein synthesis in dams, their offspring presented no signs of improvement in the indicators of protein synthesis. Restriction of growth observed in offspring may be due to the inhibitory effect of prolonged maternal LP diet which seems to exceed the range of recovery effect by amino acid supplementation.

A significant catch-up in liver growth of offspring was observed based on PCNA immunoblotting. In liver development process, hepatic PCNA and b-catenin expression are increased from 5 to 20 days after birth, and hematopoietic cells are observed until about 5 or 10 days after birth and then disappeared in about 25 days (Apte *et al.*, 2007). Therefore, high levels of PCNA observed in LP offspring may indicate retarded hepatocyte maturation. However, BCAA supplementation did not alleviate the altered liver development of offspring. Furthermore, we observed disturbed sinusoid development in the liver of the LP-O group, which were alleviated by maternal dietary supplementation of BCAA or Ala. The BCAA-O group showed a tendency about HNF4α, which is a key growth factor involved in the prenatal and postnatal liver and sinusoid development (Parviz *et al.*, 2003), which was not significant but increased as compared to LP offspring. Thus, although maternal BCAA supplementation did not prevent the delay of normal liver maturation, it is possible that the expression of Hnf4α is

related to restoration of vascular development.

In this study, maternal Ala supplementation had comparable effects to BCAA supplementation. Because BCAA supplementation was preconceived to show greater effects to improve the maternal protein status and growth of offspring than Ala supplementation, these finding was an unexpected result. As not a toxic substance, antioxidant and neurotransmitter, Ala has been frequently used as a negative control in several amino acid supplementation studies (Cao *et al.*, 2009; Ham *et al.*, 2014). However, Ala supplementation on maternal LP diet significantly increased serum albumin, leptin, hepatic total protein content and hepatic S6 expression as well as BCAA supplementation. In addition, in the PD21 offspring, the area of hepatic sinusoids of Ala-O group was significantly increased like those of BCAA group. Similarly, positive effects of both dietary leucine and alanine supplementation in the prevention of obesity in mice fed a high-fat diet were observed (Freudenberg *et al.*, 2013). In addition, both BCAA and alanine supplementation normalized serum glutamine levels which were significantly higher in LP group compared with NP group of PP21 dams. Metabolized BCAA in muscle synthesizes alanine and glutamine (Holeček, 2002), and these interconversions of BCAA, glutamine and alanine may have influenced to offset the specific effects of BCAA and Ala. Also, BCAA diet (15.9 g of nitrogen/kg diet) and Ala diet (16.8 g of nitrogen/kg diet) had similar nitrogen content, which were significantly higher than those of LP diet (12.57 g of nitrogen/kg diet)

in this study. Therefore, the reverse effects of BCAA supplementation on LP dams and offspring from LP dams may not be BCAA specific manner, but the effects of increased nitrogen supply compared with LP group. Overall, these results suggest that negative control group which is isonitrogenous with experimental group is needed for evaluating the specific effects of amino acids such as BCAA in further studies.

This study is, to our knowledge, the first study that examined the effects of maternal BCAA supplementation on dams fed an LP diet and weaned offspring. Serum and hepatic protein levels of dams and area of liver sinusoids of offspring, which were inhibited by maternal LP diet consumption, were improved both by maternal BCAA and Ala supplementation. In conclusion, BCAA did not showed specific recovery effects on reduced protein synthesis in dams fed an LP diet and retarded liver development in offspring from LP dams. Additional nitrogen supply may be responsible for increasing hepatic protein synthesis in both dams and offspring and alleviating distorted liver development of offspring from dams fed an LP diet. Further studies with metabolically challenged adult offspring would be needed to investigate the long-term effects of BCAA supplementation in maternal diet.

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국문 초록

어미 마우스의 분지아미노산 보충 섭취가 저단백 식이를 섭취한 어미의 단백질 대사와 자손의 간 발달에 미치는 영향

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모체의 영양 상태는 자손의 성장과 밀접하게 연관되어 있다. 모체 저단백 (low-protein, LP) 식이는 mammalian target of rapamycin (mTOR) 신호전달기전의 활성을 감소시켜 어미에게 이화 상태를 유발하는 한편, 태아의 간 발달을 구조적, 기능적으로 저해하여 성인 자손의 심혈관계 질환, 비만 및 비알코올성 간질환의 발병에 영향을 주는 것으로 보고된다. 분지아미노산 (branched-chain amino acid, BCAA)은 특히 류신을 통해 mTOR 신호전달기전을 활성화시켜 단백질 합성을 직접 촉진하는 필수아미노산이다. 그러나 모체 LP 연구에서 어미의 BCAA 보충 섭취가 수유기 어미의 단백질 대사와 자손의 간 발달이 받는 영향에 대한 연구는 미흡한 실정이다. 따라서, 본 연구에서는 임신 전부터 수유기까지의 어미의 BCAA 보충 섭취가 모체 LP에 의해 저해된 어미의 단백질 대사와 자손의 간 발달에 미치는 효과를 보고자 하였다. 암컷 CD-1 마우스는 임신 전 2주, 임신기 및 수유기 동안 대조 식이 (20% casein), 저단백 식이 (10% casein), 저단백 식이에 2.1% BCAA를 보충한 식이 또는 BCAA군에 대한 질소 대조군으로서 저단백 식이에 2.1% 알라닌 (Ala)을 보충한 식이를 공급받았다. 어미와 수컷 자손 마우스는 출생 후 3주에 희생하여 기관 무게, 혈청 및 간 조직을 분석하였다. 선행 연

구에서 보고된 것과 같은 모체 LP 모델이 임신기부터 잘 유도되었는지 확인하기 위해, 출생 후 2-3일차 어미와 자손도 추가로 희생하여 조직 무게와 혈청만을 분석하였다.

연구 결과, 출생 후 2-3일차 어미와 자손은 모체의 BCAA 보충 섭취에 따른 측정 지표의 변화가 없었다. 그러나 출생 후 3주 어미는 혈청 렙틴 농도와, 대조군에 비해 LP군에서 유의적으로 낮았던 혈청 알부민 농도 및 간 단백질 농도가 BCAA 또는 Ala 보충 섭취에 의해 유의적으로 증가되었다. 어미의 렙틴 농도는 어미의 간 내 총 단백질 농도 (r = 0.523, P = 0.038)와 유의적인 상관관계를 가져, BCAA 및 Ala 보충에 의한 렙틴 증가와 간 내 단백질 농도 간 연관성을 시사하였다. 어미의 혈청 BCAA 농도는 희생시점이나 실험식이에 따른 차이가 없었으나, 모체의 BCAA 또는 Ala 보충 섭취는 대조군에 비해 LP군에서 유의적으로 낮았던 S6 단백질 발현을 유의적으로 증가시켰다. 모체 BCAA 보충은 대조군에 비해 LP군에서 유의적으로 낮았던 수유기 자손의 체중 및 기관 무게, 혈청 생화학적 지표에 대해 유의한 영향을 미치지 않았다. LP군의 수유기 자손은 대조군에 비해 간 내 proliferating cell nuclear antigen 발현이 유의적으로 높고 모세혈관 직경은 감소되었으며 초기 간 발달에 중요한 hepatocyte nuclear factor 4α 및 간 모세혈관에서 저밀도 지단백질 수송에 관여하는 low-density lipoprotein receptor의 발현이 낮아, 간 발달이 지연되었음을 시사하였다. 이에 대해 모체 BCAA 또는 Ala 보충 섭취는 간 내 모세혈관 직경을 유의적으로 증가시켰다.

결론적으로, 임신 전, 임신기 및 수유기 동안의 모체 BCAA 보충 섭취는 동일 기간의 모체 LP에 의해 저해된 어미의 단백질 대사와 자손의 간 발달에 대해 특이적인 회복 효과를 보이지 않았다. LP군에 대해 BCAA, Ala군에서 동일하게 나타난 어미 및 자손의 지표 회복은 아미노산 보충에 의한 질소 증가에 의해 유도된 것으로 사료된다.

주요어: BCAA 보충, 모체 저단백 식이, 단백질 대사, 간 발달, 자손 쥐
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