A THESIS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD AND NUTRITION

Effects of branched-chain amino acid supplementation in dams fed a low-protein diet on muscle growth and mitochondrial oxidative capacity in obese offspring

저단백식이를 섭취한 어미의 분지아미노산 보충섭취가 비만한 자손의 근육 발달과 미토콘드리아 산화 능력에 미치는 영향

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Abstract

Effects of branched-chain amino acid supplementation in dams fed a low-protein diet on muscle growth and mitochondrial oxidative capacity in obese offspring

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Maternal low protein (LP) diet has been reported to have a negative impact on the onset of metabolic diseases in adulthood, which is related to growth retardation during fetal development. Branched-chain amino acids (BCAA), especially leucine, activates mechanistic target of rapamycin pathway that stimulate protein synthesis, and it is known that the circulating level of BCAA is reduced in the dams fed an LP diet. It suggests that maternal BCAA supplementation could relieve the growth retardation and this restoration could affect muscle oxidative capacity through alterations of muscle fiber distribution, and finally cause changes in response to high-fat diet. To test, female ICR mice were fed a normal protein (NP, 20% casein), low protein (LP, 10% casein), LP with 2% BCAA or 2% alanine (Ala) diet two weeks prior to mating and throughout gestation and lactation. Male offspring were challenged with high fat

(45% kcal from fat) diet after weaning to 25 weeks of age. Gastrocnemius muscle weight and Pax7 mRNA expression level, a satellite cell marker, were decreased by maternal LP feeding, but were reversed by BCAA and Ala supplementation. Furthermore, maternal LP consumption reduced total ribosomal protein S6 level, a marker of ribosome biogenesis, which was increased in the LP+BCAA and LP+Ala groups. However, the markers related to the high-fat diet induced atrophy, such as ER stress, were not changed by maternal LP diet. HOMA-IR, an index of systemic insulin resistance, was significantly lower in the LP group compared with the NP group, but the effect of maternal BCAA supplementation was not observed. Moreover, there were no changes in the mitochondrial oxidative capacity of gastrocnemius and soleus muscle. In conclusion, maternal BCAA supplementation has protective effects on the damaged muscle growth and these effects were mediated by increasing satellite cell number and ribosome biogenesis. However, the specific effect of BCAA on muscle growth was not observed.

Keyword: BCAA, low protein diet, mouse offspring, high-fat diet, muscle, mitochondria

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List of Abbreviations

Ala: alanine

ATF4: activating transcription factor 4

BCAA: branched-chain amino acid

CD68: cluster of differentiation 68

CHOP: C/EBP homologous protein

COX: cytochrome c oxidase subunit

CPT1B: carnitine palmitoyltransferase 1B

ER stress: endoplasmic reticulum stress

GAS: gastrocnemius

GRP78: 78kDa glucose regulatory protein

HO-1: heme oxygenase 1

HOMA-IR: homeostasis model assessment for insulin resistance

LP: low protein

MCP1: monocyte chemotactic protein 1

MuRF1: muscle-specific ring finger protein 1

MYH: myosin heavy polypeptide

NRF1: nuclear respiratory factor 1

PAX7: paired box 7

RPL19: ribosomal protein l19

SOL: soleus

TFAM: transcription factor A, mitochondrial

1. Introduction

1.1. Perinatal nutritional status and maternal low-protein model

Nutritional status during perinatal period has been reported to affect the risk of metabolic disease in adulthood as well as fetal development (Hales *et al.* 1992, Ozanne *et al.* 2002). When the supply of nutrients during fetal development is limited, most energy sources are assigned preferentially to brain development. As a result, other tissues, such as liver, pancreas and skeletal muscle, have an adaptive programming to survive in this environment. When this detrimental nutritional status disappears after birth, a discrepancy between perinatal programming and postnatal environment is increased, inducing excessive catch-up growth through fat accumulation. Even if the offspring experienced the catch-up growth, the muscle growth retardation is not restored (De Blasio *et al.* 2007). Consequently, the body composition is weighed towards fat accumulation, resulting in decreased energy metabolism and glucose homeostasis, which increase the risk of the metabolic syndrome (Reichling *et al.* 2000, Thorn *et al.* 2011).

Maternal low-protein (LP) diet model is one of the well-established model to cause intrauterine growth restriction. Maternal LP diet is known to induce changes in offspring's growth, risk of metabolic syndrome, and predisposition of senile to sarcopenia (Jahan-Mihan *et al.* 2015, Sayer *et al.* 2010). The changes observed in the offspring of dams fed an LP diet have

been reported differently depending on the period of feeding a protein restricted diet (Agnoux *et al.* 2014, Alexandre-Gouabau *et al.* 2011). It has been confirmed that feeding an LP diet during gestation and lactation has the more deleterious effect on offspring growth rather than feeding only during gestation (Zambrano *et al.* 2006). However, in terms of metabolic disease, maternal protein restriction during only gestation leads to excessive catch up growth and causes the metabolic disease in adulthood (Han *et al.* 2012).

Furthermore, some studies have reported that changes in offspring growth and susceptibility to metabolic disease could be accelerated by high-fat diet after weaning (Whitaker *et al.* 2012, Wilson *et al.* 1997). When high-fat diet is provided to adult offspring, followed by perinatal protein restriction, metabolism are changed in several tissues, such as skeletal muscle (Claycombe *et al.* 2015), brown and white adipose tissue (Dumortier *et al.* 2017, Xie *et al.* 2017) and liver (Souza-Mello *et al.* 2007).

1.2. Effects of maternal low-protein diet on muscle development of offspring

Like any other organs, muscle growth (Cabeco *et al.* 2012, de Melo *et al.* 2011, Mallinson *et al.* 2007) and fiber development (Confortim *et al.* 2015, Leandro *et al.* 2012) are known to be affected by maternal LP diet. In protein restricted environment, the number of myofiber and satellite cell of fetus is decreased (Yates *et al.* 2012).

Satellite cell is a small mononuclear cell located within the basal lamina of muscle fiber (Le Grand *et al.* 2007). This type of cell usually remains in a quiescent state, but becomes activated when a stress, like injury or exercise, is given. At the activation phase, satellite cell can perform asymmetric divisions for the generation of myogenic lineage and self-renewal, resulting in constant pool of satellite cell (Dumont *et al.* 2015). The contents of satellite cell are established by postnatal day 21 and rarely change afterwards (White *et al.* 2010), and it plays an important role in the regulation of muscle growth and repair during postnatal growth (Moss *et al.* 1971). They imply that the effect of maternal diet on satellite cell formation during fetal development in relation to change in muscle mass in adulthood. Since, the PAX7 gene is expressed in the quiescent satellite cell (Zammit *et al.* 2006) and the function of the satellite cell in adult skeletal muscle relies on the expression of this gene, it is currently accepted as a most reliable marker for satellite cell (von Maltzahn *et al.* 2013).

In addition, maternal LP diet could induce the alternation of muscle fiber distribution and mitochondrial oxidative capacity in offspring (Jousse *et al.* 2014). Skeletal muscle is a heterogeneous tissue composed of several fiber types, which differ in mitochondrial content. Type I fibers, a slow-twitch fiber, contain more mitochondria than other types and have a higher lipid oxidation capacity. Whereas Type IIb fibers, known as fast-twitch fiber, have higher glucose oxidation capacity and Type IIa fibers represent the intermediate characteristic of these two fibers (Schiaffino *et al.* 2011, Wigmore *et al.* 1998). Because of the different oxidative capacity, the distribution of muscle fiber affects the onset of diseases such as obesity and insulin resistance (Hickey *et al.* 1995). The gastrocnemius (GAS) muscle and soleus (SOL) muscle are located in the calf muscle and have different muscle fiber composition (Bloemberg *et al.* 2012). The GAS muscle is mainly composed of Type IIa and IIb fibers, and the SOL muscle is mainly composed of Type I fibers (Shortreed *et al.* 2009). It has been reported that offspring from dams fed an LP diet have the lower density of Type I fiber (da Silva Aragao *et al.* 2014, Leandro *et al.* 2012). Although the changes in fiber distribution are different according to the various experimental conditions, such as nature of diet and feeding period (Cabeco *et al.* 2012, Mallinson *et al.* 2007), perinatal nutritional status is one of the important factor in determining fiber type.

1.3. Effects of high-fat diet on muscle mass and mitochondrial function

Chronic consumption of high-fat diet causes metabolic changes in several tissues, including skeletal muscle (Buettner et al. 2007). High-fat diet induces endoplasmic reticulum stress (ER stress) through the increase of oxidative stress and inflammation, resulting in attenuation of protein synthesis and accelerated degradation (Le *et al.* 2014, Rodriguez *et al.* 2015, Yuzefovych *et al.* 2013). Upon ER stress, Bip/glucose-regulating protein 78 (GRP78) dissociates from protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). Consequently, phosphorylation of eukaryotic translation initiation factor 2α (eIFα) and translation of activating transcription factor-4 (ATF4) are increased (Harding et al. 1999). The increased phosphorylation of eIFα inhibits the formation of translation initiation complex, and finally attenuates global protein translation. ATF4 induces transcription of C/EBP homologous protein (CHOP) involved in apoptosis and other genes related to ubiquitin-proteasome system and autophagy-lysosomal system (Bohnert et al. 2017).

High-fat diet also affects the mitochondrial oxidative capacity of skeletal muscle (Shortreed *et al.* 2009, Sparks *et al.* 2005). High-fat diet leads to increase in circulating free fatty acid level released from adipose tissue. If the amount of free fatty acid exceeds the oxidative capacity of mitochondria, lipid intermediate are produced, interrupting the insulin

signaling pathway and damaging mitochondrial function (Rachek 2014, Turner *et al.* 2013). In addition, the increase in ROS production leads to the accumulation of oxidized acylcarnitine, increasing H_2O_2 emission (Anderson et al. 2009), and ultimately impairs mitochondrial function.

Conversely, the possibility that mitochondrial dysfunction is responsible for insulin resistance has been suggested (Chanseaume *et al.* 2009), implicating that changes in the oxidative capacity by maternal diet may affect the ability to cope with a high-fat diet.

1.4. Effects of BCAA supplementation on protein synthesis

Branched-chain amino acids (BCAA) refer to leucine, isoleucine, and valine. These amino acids, especially leucine, stimulate protein synthesis through mechanistic target of rapamycin (mTOR) pathway (Anthony *et al.* 2001, Crozier *et al.* 2005). Leucine supplementation in LP diet causes increasing of protein synthesis in muscle through enhanced phosphorylation of mTOR pathway signaling molecules, such as 4E-BP1, S6K1, and eIF4G (Murgas Torrazza *et al.* 2010). Although isoleucine and valine could not affect mTOR signaling activity or muscle protein turnover (Escobar *et al.* 2006), supplementation of leucine alone may induce the imbalance of BCAA in the circulation, masking the positive effects of leucine. Therefore, the supplementation of these three amino acids together is desirable to confirm the effects of leucine in protein synthesis (Wu 2009).

The main features observed in dams fed an LP diet are the decreasing in circulating BCAA level (Bhasin *et al.* 2009) and mTOR signaling pathway (Rosario *et al.* 2011), which induce the growth restriction of placenta and mammary gland and reduction of amino acid transporter. As a result, BCAA availability and mTOR signaling in the fetus is also reduced (Eckert *et al.* 2012, Teodoro *et al.* 2012). Because of the stimulating effect of BCAA on protein synthesis, the supplementation of these amino acids in dams fed an LP diet could restore the growth retardation of the fetus. It may also affect muscle fiber development of fetus, which is occurred during fetal development. However, there is no study yet about the maternal

BCAA supplementation on adult offspring, especially related to skeletal muscle growth and metabolic capacity in response to the high-fat diet challenge.

1.5. Aim of this study

This study was aimed to investigate the effects of BCAA supplementation in dams fed an LP diet on muscle of adult offspring. Our hypothesis is that maternal diet has influences on the muscle development of fetus, resulting changes in muscle mass and fiber distribution. Additionally, it was also investigated whether changed muscle development by maternal diet could affect the response in muscle of offspring in obesogenic environment.

2. Materials and Methods

2.1. Animals and diets

Virgin female ICR mice were obtained from Orient Bio Co. (Korea) and were housed in a temperature and humidity-controlled room with a 12h dark/light cycle. After an adaptation period with a chow diet, mice were randomly allocated into 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% BCAA, the same amount contained in the NP diet, and the Ala group was fed an LP diet with 2% alanine, one of the non-toxic amino acid. All groups were isocaloric and alanine was used as a control supplying equivalent quantities of nitrogen with BCAA group. 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. The composition of diet is described in **Table 1**. Each experimental diets were provided *ad libitum* for 2 weeks before mating and throughout pregnancy and lactation (**Figure 1**).

After delivery, litters were standardized to ten pups with six males and four females wherever possible. At 3 weeks of age, male offspring were fed a low-fat diet (10% kcal from fat, #D12450B, Research diets Inc., USA) or high-fat diet (45% kcal from fat; #D12451, Research Diets Inc., USA) for 22 weeks. The offspring of dams fed a normal protein diet were divided

into two groups, one group received a low-fat diet and the other group received a high-fat diet. The low-fat fed group was established to confirm that whether diet-induced obesity is well occurred. Therefore, this group used only for comparison with the NP group in body weight, organ weight, and serum parameters related with obesity. The offspring of dams fed an LP, LP+BCAA, and LP+Ala diet were provided high-fat diet to find out whether metabolic programming caused by maternal LP diet could affect the response to high-fat diet challenge. The body weight of offspring was measured weekly and food intake was recorded three times a week. At the end of experiment day, mice were sacrificed by using Zoletil® (Virbac, France) and Rompun® (Bayer Korea, Korea) in the morning after 14 h overnight fasting. Two offspring per each dam were further analyzed. Blood were collected by cardiac puncture and used in serum biochemical analyses. GAS muscle and SOL muscle were collected and kept at -80°C until use. All the experiments were approved by Seoul National University Institutional Animal Care and Use Committee (permission number: SNU-151019-6-2).

Table 1. Composition of experimental diet of dams

Composition (g/kg diet)	Diet			
	NP	LP	LP+BCAA	LP+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-Cysteine	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
TBHQ	0.014	0.014	0.014	0.014
Total	1002.5	1004.4	1004.4	1004.4

[1] CA160030 (Harlan, USA)

[2] TD.94046 (Harlan, USA)

[3] TD.94047 (Harlan, USA)

NP, normal protein; LP, low protein; LP+BCAA, low protein supplemented with BCAA; LP+Ala, low protein supplemented with alanine

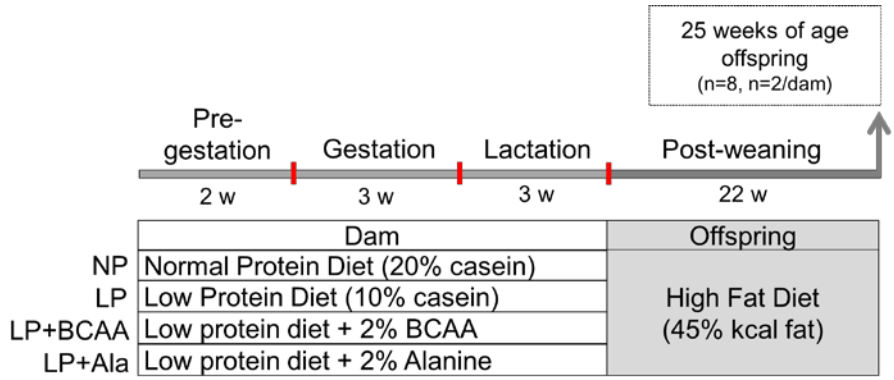

Figure 1. Overview of the study design.

2.2. Serum biochemical analysis

Serum IGF-1 level of dam was determined by mouse/rat IGF-1 ELISA kit (R&D systems, Inc., USA). The fasting serum glucose level was measured with colorimetric method using commercial kit (Asan Pharmaceutical Co., Korea) and serum insulin level was determined by using a mouse insulin ELISA kit (Shibayagi Co., Japan). Two offspring per each dam were used for serum analyses, so eight offspring per group were included. The insulin resistance index was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) with the following formula: serum glucose (mmol/L) × serum insulin (mU/L)/22.5.

2.3. Total protein extraction and immunoblotting

GAS muscle from two offspring of each dam were pooled for total protein extraction. Pooled samples were homogenized by using a lysis buffer [150 mmol/L NaCl, 50 mmol/L Hepes-KOH (pH 7.5), 2.5 mmol/L EGTA (pH 8.0), 1 mmol/L EDTA (pH 8.0), 1 mmol/L NaF, 10 mmol/L β-glycerophosphate, 0.1 mol/L Na_3VO_4, 0.1% Tween-20, 10% glycerol, 1 mmol/L DTT and the protease inhibitor cocktail (sigma, USA)]. The concentration of protein were measured by using Bradford protein assay kit (Bio-Rad, USA). Equal amounts of protein were boiled for 5 minutes and loaded into SDS-PAGE gel. Separated protein were transferred to polyvinylidene fluoride membrane and this membrane was incubated with primary antibody and secondary antibody diluted in 5% BSA or 5% skim milk: KDEL/GRP78 (ADI-SPA-827, Enzo Life Sciences, USA), p-S6 (#2211, Cell Signaling Technology, USA), Total S6 (sc-74459, Santa Cruz Biotechnology, USA), Ub (sc-8017, Santa Cruz Biotechnology, USA), GAPDH (sc-32233, Santa Cruz Biotechnology, USA). To detect the protein band, HRP substrate (Millipore, USA) were treated and this band visualized by X-ray film (Fuji, Japan). After visualization, the intensity of bands was quantified through Quantity one software (Bio-Rad, USA).

2.4. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from GAS muscle and SOL muscle, and two offspring of each dam were pooled for RNA extraction. Total RNA was extracted using RNAiso Plus (Takara, Japan). The quality of RNA was verified using spectrophotometer at 260/280nm absorbance. cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen, USA). StepOne™ Real Time PCR system (Applied Biosystems, USA) was used for measuring relative mRNA expression level. SYBR™ Green PCR Master Mix (Applied Biosystems, USA) and specific primer were added to cDNA. As a loading control, RPL19 was also amplified. Relative mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ method and the sequences of primer used in experiment were presented at **Table 2**.

Table 2. Quantitative real-time PCR primer sequences

Gene		Sequence (5′→3′)
ATF4	Forward	TCGATGCTCTGTTTCGAATG
	Reverse	GGCAACCTGGTCGACTTTTA
CD68	Forward	GCACAGCCAGCCCTACGA
	Reverse	GAGCTGGTGTGAACTGTGACATTT
CHOP	Forward	AGCTGGAAGCCTGGTATGAGGA
	Reverse	AGCTAGGGACGCAGGGTCAA
COX3	Forward	CAAGGCCACCACACTCCTAT
	Reverse	ATTCCTGTTGGAGGTCAGCA
COX4-1	Forward	ACTGCGCTCGTTCTGATTTGG
	Reverse	CATTCGCTTGGTCTGCATGG
COX4-2	Forward	CTGCCCGGAGTCTGGTAATG
	Reverse	CAGTCAACGTAGGGGGTCATC
CPT1B	Forward	CCAGATCTGCATGTTTGACC
	Reverse	TGCTGGAGATGTGGAAGAA
GRP78	Forward	TTCAGCCAATTATCAGCAAACTCT
	Reverse	TTTTCTGATGTATCCTCTTCACCAGT
HO-1	Forward	CCTCACTGGCAGGAAATCATC
	Reverse	CCTCGTGGAGACGCTTTACATA
MCP1	Forward	TGATCCCAATGAGTAGGCTGG
	Reverse	ATGTCTGGACCCATTCCTTCTTG

MuRF1	Forward	ACGAGAAGAAGAGCGAGCTG
	Reverse	CTTGGCACTTGAGAGGAAGG
MYH2	Forward	AAAGCTCCAAGGACCCTCT
	Reverse	AGCTCATGACTGCTGAACTCAC
MYH7	Forward	AGTCCCAGGTCAACAAGCTG
	Reverse	TTCCACCTAAAGGGCTGTTG
NRF1	Forward	ATCCGAAAGAGACAGCAGACA
	Reverse	TGGAGGGTGAGATGCAGAGTA
PAX7	Forward	GACGACGAGGAAGGAGACAA
	Reverse	CGGGTTCTGATTCCACATCT
TFAM	Forward	GAAGGGAATGGGAAAGGTAGA
	Reverse	AACAGGACATGGAAAGCAGA
RPL19	Forward	TCAGGCTACAGAAGAGGCTTGC
	Reverse	ATCAGCCCATCCTTGATCAGC

ATF4: activating transcription factor 4, CD68: cluster of differentiation 68, CHOP: C/EBP homologous protein, COX: cytochrome c oxidase subunit, CPT1B: carnitine palmitoyltransferase 1B, muscle, GRP78: 78kDa glucose-regulated protein, HO-1: heme oxygenase 1, MCP1: monocyte chemotactic protein 1, MuRF1: muscle-specific ring finger protein 1, MYH: myosin heavy polypeptide, NRF1: nuclear respiratory factor 1, PAX7: paired box 7, TFAM: transcription factor A, mitochondrial, RPL19: ribosomal protein L19

2.5. Statistical analysis

All data were analyzed using SPSS software (Ver. 22.0, SPSS Inc., USA). To confirm differences in all groups, one-way analysis of variance (ANOVA) method was used. Where appropriate, post hoc comparisons were made by Duncan's multiple range test. The data were expressed as mean ± SEM and differences were considered significant at $p < 0.05$. Correlation between two variables were determined by Pearson's correlation coefficient.

3. Results

3.1. Effect of BCAA supplementation in dams fed an LP diet on body weight and muscle weight of adult offspring

To determine whether diet-induced obesity occurred well, the offspring of dams fed a normal protein diet was divided into low-fat fed and high-fat fed group. High-fat diet significantly increased body weight (NP/LF: 55.75 ± 0.96, NP/HF: 62.12 ± 2.74) and HOMA-IR (NP/LF: 5.0 ± 0.8, NP/HF: 13.8 ± 2.4), suggesting that obesity was well induced by high-fat diet.

Final body weight of the NP group was higher than that of the LP group and there was no effect of maternal BCAA and Ala supplementation (**Figure 2A**). Whereas the GAS muscle weight was decreased in the LP group compared with the NP group and it increased in the LP+BCAA and LP+Ala group compared with the LP group (**Figure 2B**). The weight of epididymal fat tended to increase in the LP group compared to the NP group, and tended to decrease in the LP+BCAA and LP+Ala group compared to the LP group (p=0.068) (**Figure 2C**).

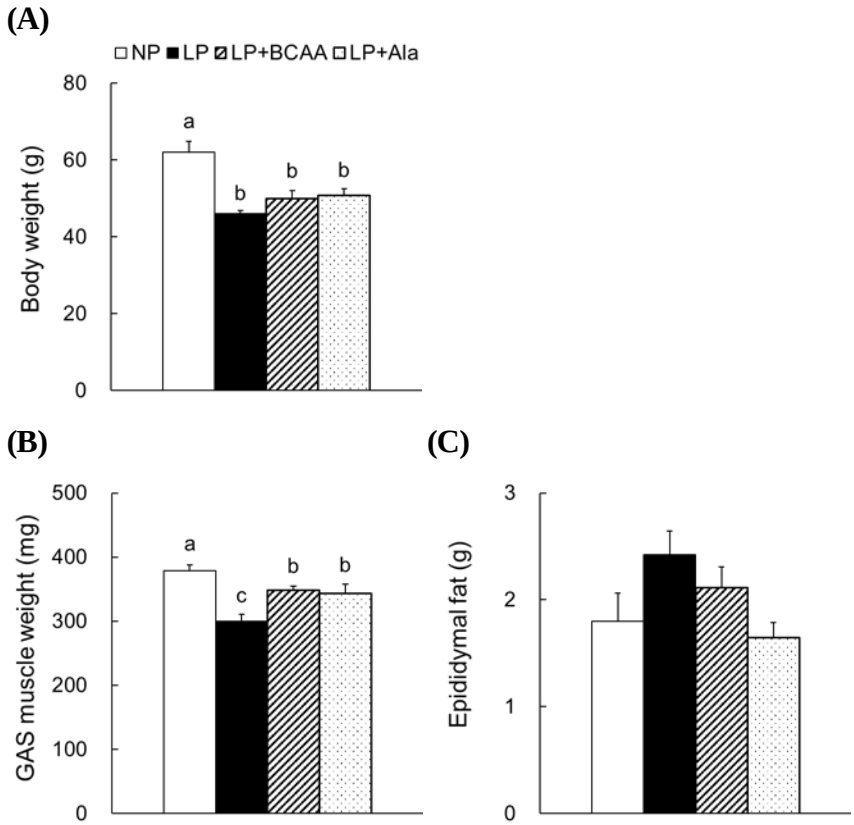

Figure 2. Effects of BCAA supplementation in dams fed an LP diet on body weight and organ weight of adult offspring.

(A) Body weight, (B) GAS muscle weight and (C) epididymal fat weight of adult offspring. Data are presented as mean ± SEM (n=8). Bars with different superscripts are significantly different (one-way ANOVA with Duncan's multiple-comparison test, $p < 0.05$).

3.2. Effect of BCAA supplementation on biochemical parameters in dams

Protein restriction is known to induce the changes in serum glucose, amino acids, and growth hormone in dams, resulting changes in fetal growth (Bhasin *et al.* 2009, Moretto *et al.* 2011). In our previous study, serum glucose, serum total protein and albumin level were decreased in dams fed an LP diet, but there was no effect of BCAA supplementation (Choi 2017). To find out the factors inducing increase of muscle mass in the LP+BCAA and LP+Ala group, serum IGF-1, important growth hormone in fetal growth (Agrogiannis *et al.* 2014), was measured. IGF-1 level tended to decrease in the LP group compared to the NP group (p=0.056), but the effect of BCAA supplementation was not observed (**Figure 3**).

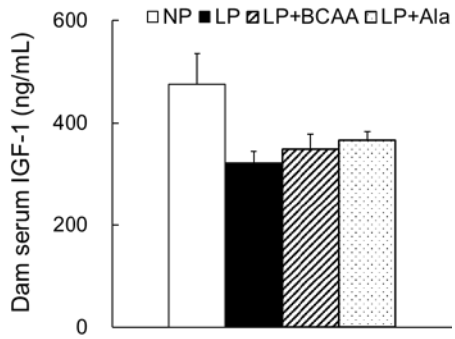

Figure 3. Effects of BCAA supplementation on serum IGF-1 level in dams.

Serum IGF-1 level was measured using ELISA kit in dams. Data are presented as mean ± SEM (n=4).

3.3. Effect of BCAA supplementation in dams fed an LP diet on satellite cell number in the skeletal muscle of adult offspring

To confirm the effect of maternal diet on satellite cell number in skeletal muscle of offspring, the mRNA expression level of PAX7, as a marker of satellite cell (Messina *et al.* 2009), was measured. The PAX7 gene expression was lower in the LP group compared with the NP group, and was higher in the LP+BCAA and LP+Ala group compared to the LP group (**Figure 4A**). The expression level of this gene has positive correlation with GAS muscle weight (r=0.53, p=0.043), suggesting that differences in adult muscle mass might be due to differences in the satellite cell number produced in early development stage (**Figure 4B**).

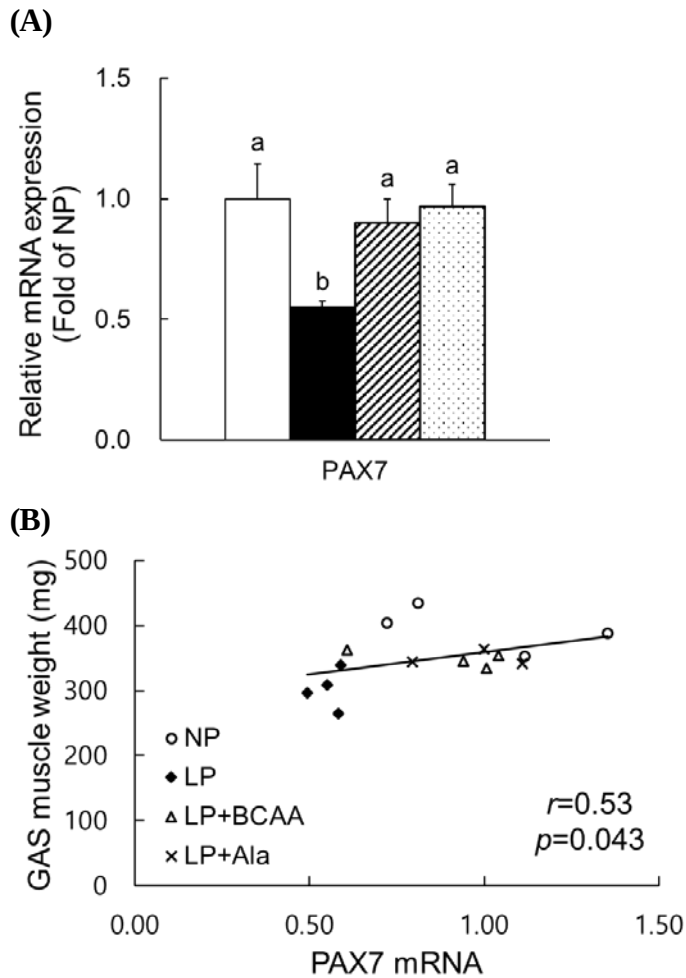

Figure 4. Effects of BCAA supplementation in dams fed an LP diet on satellite cell marker in the GAS muscle of adult offspring.

(A) Relative mRNA expression of PAX7 was measured by real-time PCR and normalized with RPL19 gene. Data are presented as mean ± SEM (n=3-4). Bars with different superscripts are significantly different (one-way ANOVA with Duncan's multiple-comparison test, $p < 0.05$). (B) Correlation between GAS muscle weight and mRNA expression level of PAX7 in the GAS muscle. Pearson's correlation coefficient, *r* and *p*-value are indicated.

3.4. Effect of BCAA supplementation in dams fed an LP diet on protein synthesis pathway in the skeletal muscle of adult offspring

To determine whether the change of muscle mass was affected by protein turnover in postnatal period, the changes in protein synthesis and degradation pathway were confirmed. Ribosomal protein S6 is involved protein translation (Ruvinsky *et al.* 2006) and ribosome biogenesis, represented as an expression level of total S6, is important for muscle hypertrophy (Chaillou *et al.* 2014). The phosphorylation level of ribosomal protein S6 related with translational efficiency was not changed by maternal diet. However, the expression level of total S6 was significantly lower in the LP group and higher in the LP+BCAA and LP+Ala group compared to the LP group (**Figure 5**).

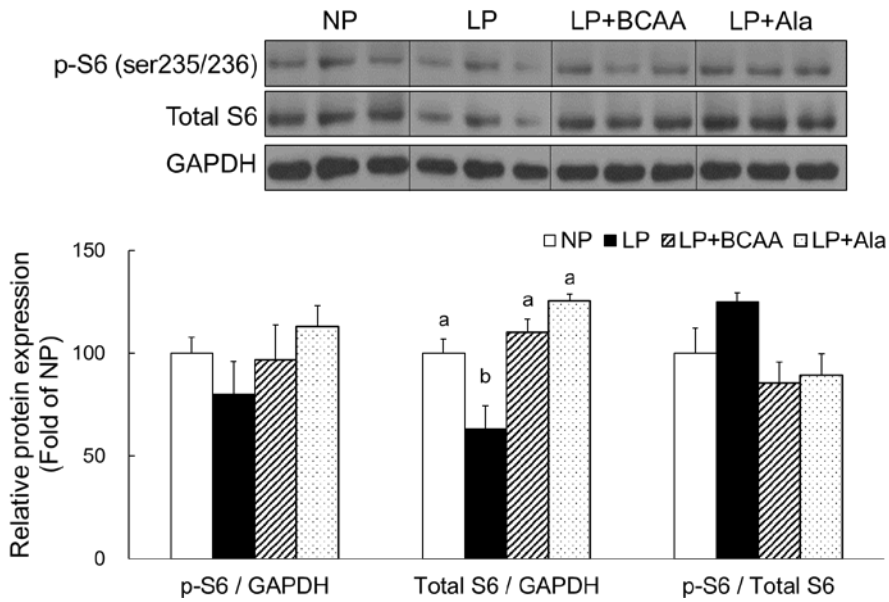

Figure 5. Effects of BCAA supplementation in dams fed an LP diet on ribosomal protein expression in the GAS muscle of adult offspring.

Protein levels of p-S6, total S6 were determined by immunoblotting and were normalized with GAPDH. Data are presented as mean ± SEM (n=3). Bars with different superscripts are significantly different (one-way ANOVA with Duncan's multiple-comparison test, $p < 0.05$).

3.5. Effect of BCAA supplementation in dams fed an LP diet on ubiquitination pathway in the skeletal muscle of adult offspring

Protein degradation pathway is also involved in the regulation of muscle mass, and ubiquitin-proteasome system is major proteolysis system involved in degradation of many structural proteins (Cao *et al.* 2005). To identify whether protein degradation was involved in the change of muscle mass, the degree of ubiquitinated-protein (**Figure 6A**) and related gene expression level (**Figure 6B**) were measured. The ubiquitinated-protein expression was decreased in the LP and LP+BCAA group compared with the NP and LP+Ala group. Also, the mRNA expression of MuRF1, muscle specific E3 ligase, tended to increase in the LP+Ala group (p=0.268), but it was not significant.

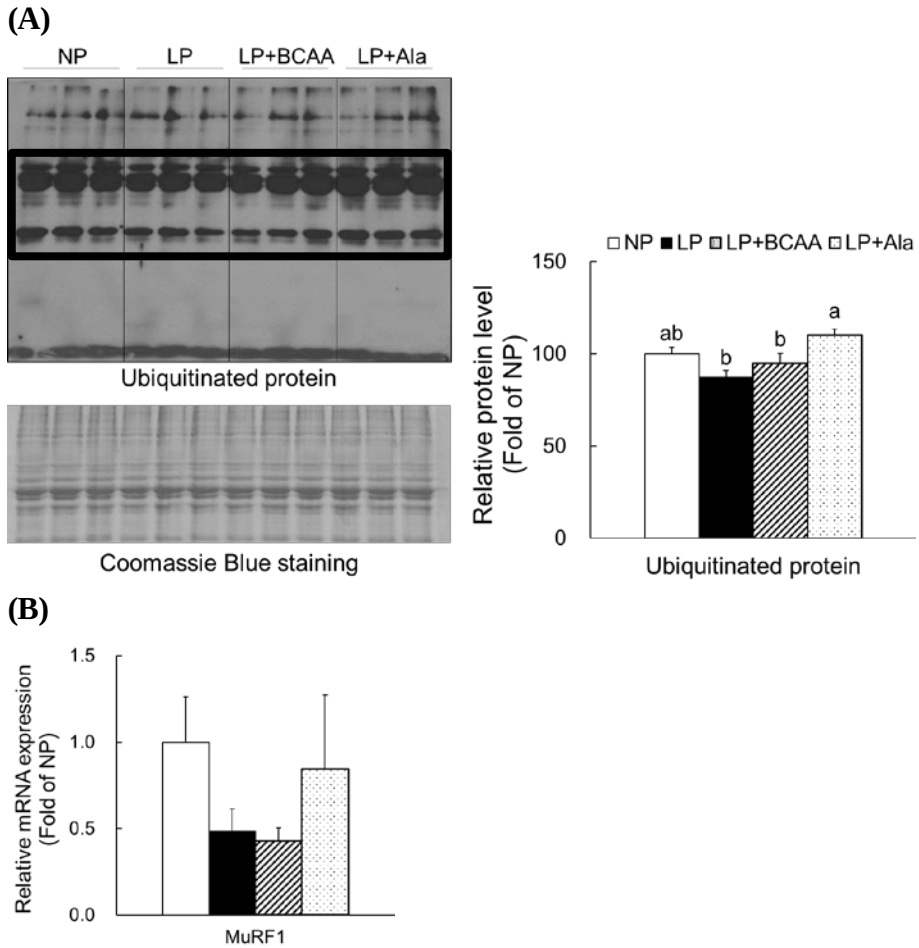

Figure 6. Effects of BCAA supplementation in dams fed an LP diet on protein ubiquitination in the GAS muscle of adult offspring.

(A) Ubiquitinated protein expression were determined by immunoblotting, and commassie blue staining gel was used for confirming that same amount of protein was loaded (n=3). The expression band in the black box was quantified for comparison of ubiquitinated protein expression. (B) MuRF1 mRNA level was determined by real-time PCR and normalized with RPL19 gene (n=3-4). Data are presented as mean ± SEM. Bars with different superscripts are significantly different (one-way ANOVA with Duncan's multiple-comparison test, $p < 0.05$).

3.6. Effect of BCAA supplementation in dams fed an LP diet on endoplasmic reticulum stress in the skeletal muscle of adult offspring

Increased ER stress is related to the inhibition of protein translation and promotion of protein degradation (Bohnert *et al.* 2017). High-fat diet induced ER stress is mediated by increased oxidative stress and inflammation, so the mRNA expression levels of the related genes, HO-1, MCP1, and CD68, were measured (**Figure 7**). However, there were no differences among groups.

The relative mRNA and protein expression levels of GRP78, an ER stress marker, were measured, but there was no difference among groups (**Figure 8**). Also, ATF4 and CHOP mRNA expression levels were not significantly changed in response to the maternal diet (**Figure 8B**).

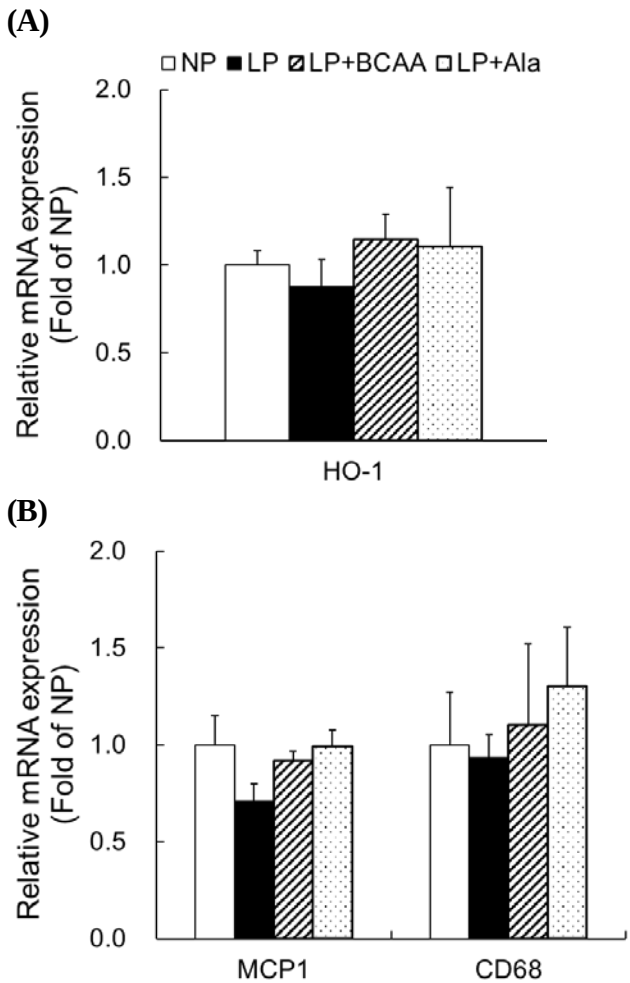

Figure 7. Effects of BCAA supplementation in dams fed an LP diet on oxidative stress and inflammation in the GAS muscle of adult offspring.

Relative mRNA expression levels of genes involved in (A) oxidative stress and (B) inflammation. The expression level of mRNA was determined using real-time PCR. Mouse RPL19 was used as an endogenous control. Data are presented as mean ± SEM (n=3-4).

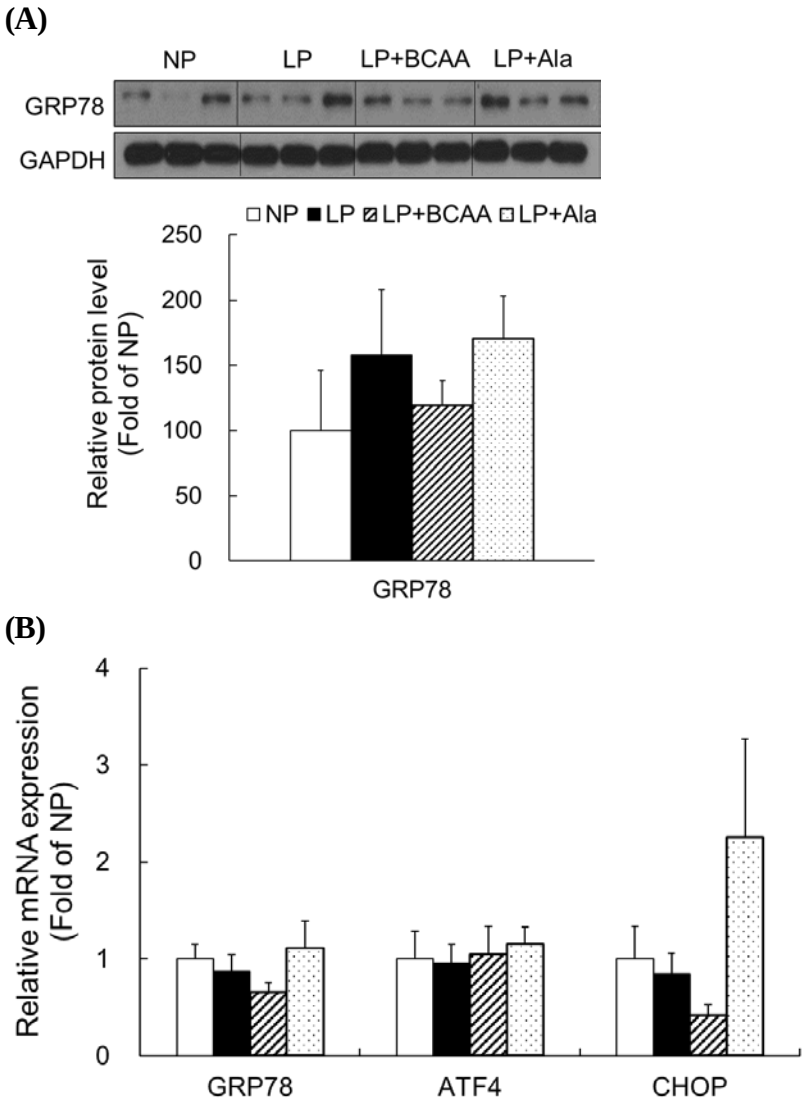

Figure 8. Effects of BCAA supplementation in dams fed an LP diet on ER stress markers in the GAS muscle of adult offspring.

(A) Protein expression level of GRP78 were determined by immunoblotting and were normalized with GAPDH (n=3). (B) Relative mRNA expression level of ER stress markers were measured by real-time PCR and normalized with RPL19 gene (n=3-4). Data are presented as mean ± SEM.

3.7. Effect of BCAA supplementation in dams fed an LP diet on whole body insulin resistance in the skeletal muscle of adult offspring

Change in whole body insulin resistance was identified to determine the effect of maternal LP diet and BCAA supplementation on metabolic changes of offspring. Fasting serum glucose (**Figure 9A**) and insulin level (**Figure 9B**) were measured and HOMA-IR (**Figure 9C**) was calculated with these two values. Fasting serum glucose level was not changed by maternal diet, but serum insulin level was significantly decreased in the LP group compare to the NP group. As a result, the HOMA-IR value was lower in the LP group compared to the NP group, indicating that the whole body insulin resistance was decreased in the LP group. However, the effect of maternal BCAA supplementation on insulin resistance was not observed.

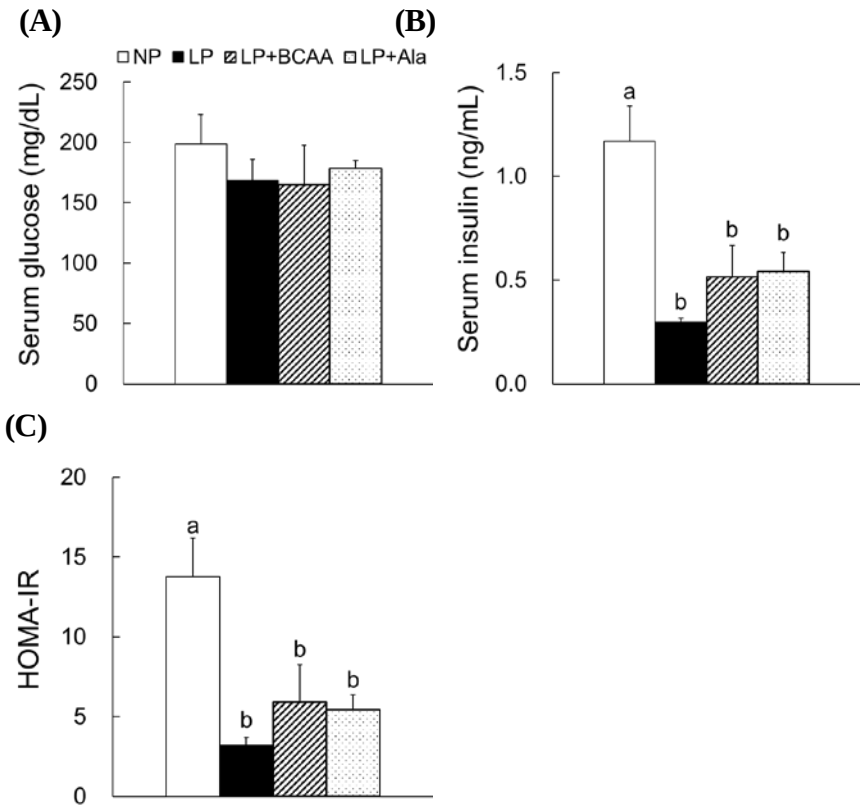

Figure 9. Effects of BCAA supplementation in dams fed an LP diet on whole body insulin resistance of adult offspring.

(A) Fasting serum glucose and (B) serum insulin were measured by using colorimetric kit and ELISA kit respectively. With these two values, (C) HOMA-IR was calculated. Data are presented as mean ± SEM (n=7-8). Bars with different superscripts are significantly different (one-way ANOVA with Duncan's multiple-comparison test, $p < 0.05$).

3.8. Effect of BCAA supplementation in dams fed an LP diet on mitochondrial oxidative capacity in the skeletal muscle of adult offspring

The effect of maternal LP diet on insulin resistance of offspring was observed in this study. There are many factors affect to the insulin resistance, and high-fat diet related change in oxidative capacity of muscle is one of them (Koves *et al.* 2008). The fatty acid oxidation capacity of muscle depends on the muscle fiber distribution and mitochondrial oxidative capacity (Stuart *et al.* 2013). Therefore, the mRNA expression levels of related genes were measured. MYH7 and MYH2 are genes primarily expressed in Type I fibers and Type IIa fibers respectively. However, there were no significant differences among all groups in GAS muscle (**Figure 10**). Also, the expression levels of NRF1 and TFAM, as markers of mitochondrial biogenesis were observed at the same level in the GAS muscle (**Figure 11A**). The differences of mRNA level of genes involved in respiratory chain (**Figure 11B**) and fatty acid oxidation (**Figure 11C**) were also not observed. Similarly, there were no significant differences in the relative expression levels of genes related to oxidative muscle fiber (**Figure 12A**) and mitochondrial oxidative capacity (**Figure 12B**) in the SOL muscle.

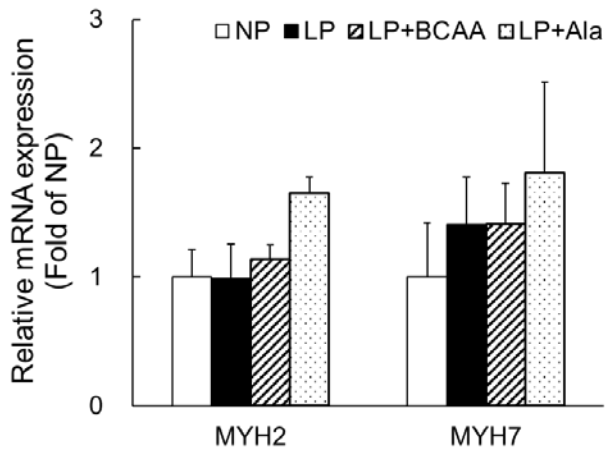

Figure 10. Effects of BCAA supplementation in dams fed an LP diet on muscle fiber phenotype in the GAS muscle of adult offspring.

The relative expression levels of muscle fiber phenotype related genes. The expression level of mRNA was determined using real-time PCR. Mouse RPL19 was used as an endogenous control. Data are presented as mean ± SEM (n=3-4).

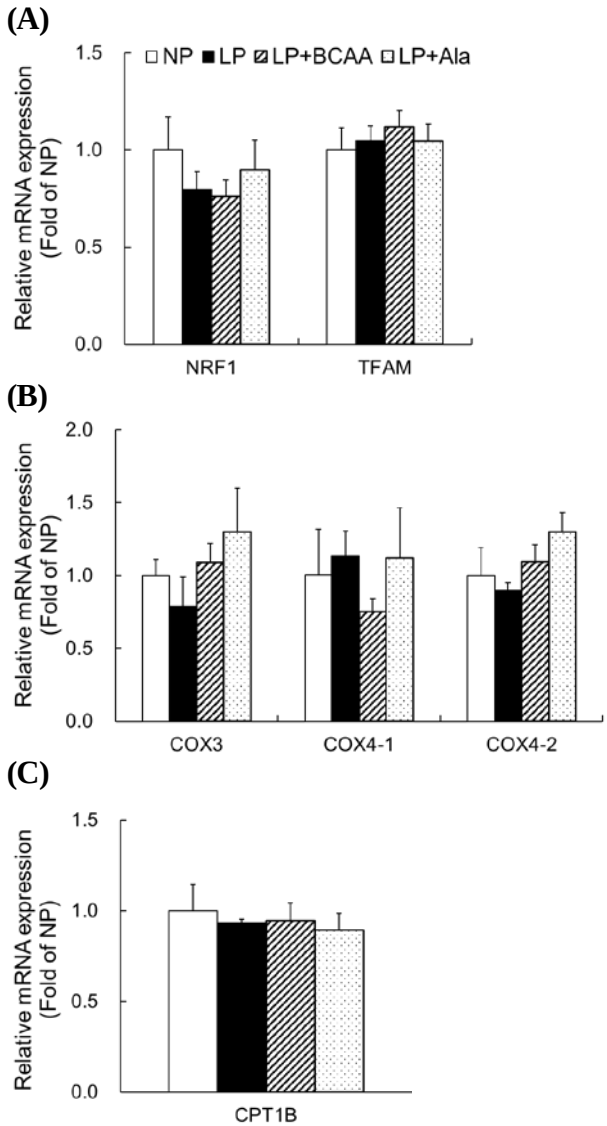

Figure 11. Effects of BCAA supplementation in dams fed an LP diet on mitochondrial oxidative capacity in the GAS muscle of adult offspring.

Relative mRNA expression levels of genes involved in (A) mitochondrial biogenesis, (B) respiratory chain and (C) fatty acid oxidation. The expression level of mRNA was determined using real-time PCR. Mouse RPL19 was used as an endogenous control. Data are presented as mean ± SEM (n=3-4).

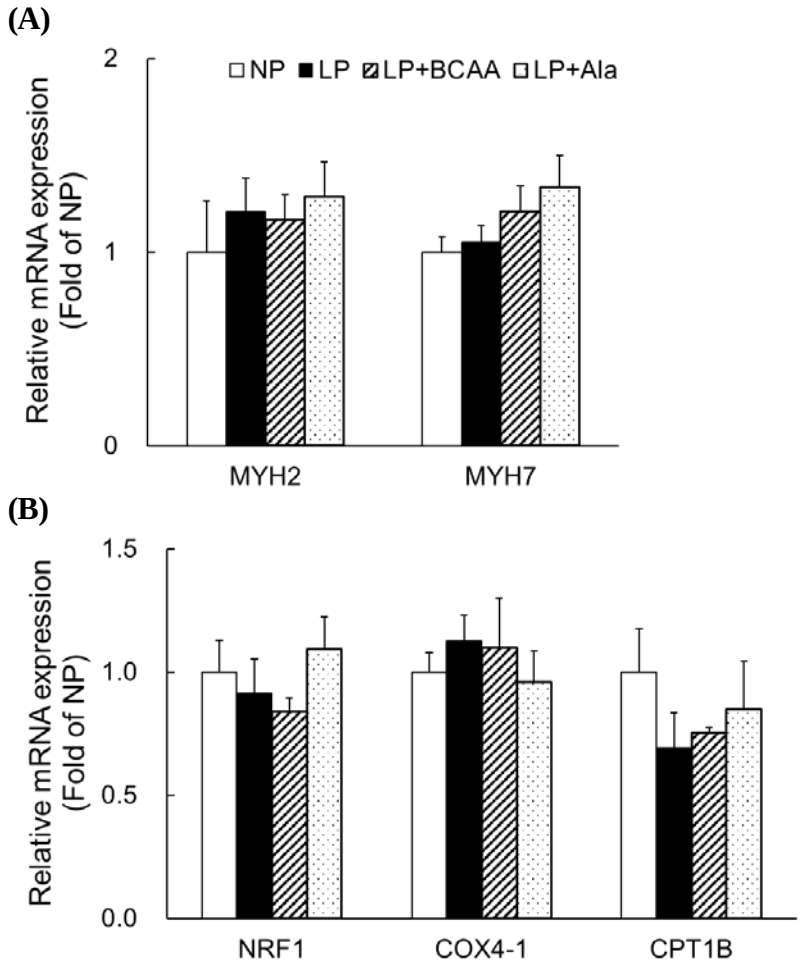

Figure 12. Effects of BCAA supplementation in dams fed an LP diet on oxidative capacity in the SOL muscle of adult offspring.

Relative mRNA expression levels of genes involved in (A) muscle fiber phenotype and (B) mitochondrial oxidative capacity. The expression level of mRNA was determined using real-time PCR. Mouse RPL19 was used as an endogenous control. Data are presented as mean ± SEM (n=3-4).

4. Discussion

Growth restriction in offspring including impaired muscle growth has been reported in maternal protein restriction model (Desai *et al.* 1996), and growth retardation is related to metabolic disease in adulthood (Desai *et al.* 1997). In consistent with previous studies, the growth of offspring from dams fed an LP diet was retarded. Maternal BCAA and Ala supplementation significantly restored decreased muscle mass, which is important to prevent the age-related muscle atrophy, known as sarcopenia (Chen *et al.* 2014). Also, the weight of epidydimal fat tended to decrease in the LP+BCAA and LP+Ala group. It shows that maternal BCAA and Ala supplementation have an impact on body composition of adult offspring.

In our previous study, serum parameters of dams were changed by LP diet without any additive effects of BCAA supplementation (Choi 2017). To find out the cause of change in offspring muscle growth, the circulating level of IGF-1, as an important factor which affect to fetal muscle growth (Brown 2014) was measured in dam. BCAA, especially leucine, is known to stimulate glucose-stimulated insulin secretion in beta cell, resulting increases in circulating IGF-1 level (Brown *et al.* 2011). Furthermore, BCAA supplementation in dams fed a calorie-restricted diet induces the improved production of hepatic IGF-1 in fetus (Chuan Zheng 2009, Mogami *et al.* 2009). However, the effect of BCAA supplementation on IGF-1 level was not observed in this study, suggesting that the muscle

weight of offspring might be affected by other factors. In particular, glucocorticoid level in dams increases during protein restriction, resulting in increased level of active glucocorticoid in fetus (Khulan *et al.* 2012). This hormone is shown to hinder muscle development through stimulation of protein degradation pathway and repression of mTOR pathway (Schakman *et al.* 2013). Because relationship between BCAA and glucocorticoid induced muscle atrophy was not fully understood, further studies are required to investigate whether BCAA supplementation is related to glucocorticoid level which involved in regulation of muscle mass.

To investigate the factors which caused changes in muscle mass, the number of satellite cell related to myogenic capacity was measured. Maternal undernutrition induced the reduction of satellite cell in adult offspring, resulting in decreased muscle mass and regeneration capacity (Woo *et al.* 2011). Maternal BCAA and Ala supplementation significantly restored the reduction of satellite cell number caused by maternal LP diet, indicating that amino acid supplementation has positive effect on satellite cell formation. In addition, the positive correlation between PAX7 mRNA expression and muscle mass suggests that increased satellite cell number contributed to increased muscle mass in adulthood.

The muscle mass is also determined by the balance between protein synthesis and degradation, but, in this study, synthesis was more important than degradation. The amount of total S6 is usually used as a marker of ribosome biogenesis, and the relationship between ribosome biogenesis

and muscle hypertrophy have been discussed in previous studies (Chaillou *et al.* 2014, Wen *et al.* 2016). Therefore, the increased expression level of total S6 in the LP+BCAA and LP+Ala group suggests that improved muscle mass may be induced by increased ribosome biogenesis. Maternal nutrient availability is related to the global transcriptional activity in fetus somatic tissue (Fleming *et al.* 2017). When dams fed an LP diet during gestation, the transcriptional activity is decreased, inducing changes in ribosomal DNA expression through increasing DNA methylation. These results show that the importance of maternal protein intake on ribosomal DNA expression of fetus. Also, it is emphasized that the importance of translational capacity on the recovery of muscle mass reduction in offspring (Fiorotto *et al.* 2014).

It was observed that HOMA-IR was significantly decreased in the LP group compared to the NP group. In general, up-regulation of MYH7, mainly expressed in Type I muscle fiber, and MYH2, expressed in Type IIa muscle fibers, have a protective effect against diet-induced obesity or insulin resistance (Bassel-Duby *et al.* 2006, Oberbach *et al.* 2006). Maternal LP diet is known to affect the muscle fiber composition of offspring. However, in this study, the oxidative muscle fiber distribution was not changed by maternal diet, resulting in same mitochondrial oxidative capacity among all groups. There are many factors affecting muscle fiber composition, such as exercise, thyroid hormone, aging and high-fat diet (Matsakas *et al.* 2009). Short-term consumption of high-fat

diet causes the increased density of Type I fiber and oxidative phosphorylation subunit (de Wilde *et al.* 2008). On the contrary, effect of chronic high-fat feeding on muscle fiber is controversial (Denies *et al.* 2014, Eshima *et al.* 2017), in part because of variable experimental conditions. Although the molecular mechanisms responsible for differences in muscle fiber phenotype by high-fat diet is not fully understood, the chronic consumption of high-fat diet may overwhelm the effect of maternal diet on muscle fiber distribution. However, the possible masking effects could not be elucidated in this study, due to the absence of low-fat fed offspring from dams fed an LP diet. To figure out whether postnatal diet has more powerful effect on muscle fiber composition rather than maternal diet, this group should be included in the future study. In addition, despite changes in whole body IR, there was no change in muscle oxidative capacity, suggesting that metabolic changes in other peripheral tissues such as liver or adipose tissue, not skeletal muscle may have been more important to insulin resistance.

To determine the specific effect of BCAA supplementation, alanine was supplemented. Because alanine is ineffective and not toxic amino acid, it has been usually used as an isonitrogenous control in studies to assess the effect of specific amino acid (Fang *et al.* 2002, Kohli *et al.* 2004). However, in this study, the Ala supplementation had a similar effect to BCAA supplementation on the muscle mass. One of the possible reason for this similarity is that the positive effect of increased nitrogen content. The

LP+Ala diet contained 16.8 g of nitrogen and LP+BCAA contained 15.9 g of nitrogen, which was higher than that of LP diet, 12.6 g of nitrogen. The protective effects of BCAA supplementation on growth retardation caused by maternal undernutrition are observed, and these effects also appears in the high-protein diet group (Mogami *et al.* 2009). This results suggest that improved nitrogen content, not specific amino acid, could alleviate adverse effect caused by malnutrition. On the other hand, in some studies, effect of leucine on postprandial muscle protein metabolism is reported, which is not observed in the alanine supplementation group (Combaret *et al.* 2005, Rieu *et al.* 2003). Taken together, it can be seen that supplementation of leucine directly to an animal has a specific effect on muscle protein metabolism, but when this amino acids were supplemented through maternal diet, there is no specific effect on muscle mass compared with alanine supplementation.

In conclusion, the present study demonstrated that maternal BCAA supplementation to an LP diet has positive effects on muscle growth retardation in adult offspring, and it may be through the improvement in maintenance of satellite cell pool and ribosome biogenesis. However, the specific effect of BCAA was not observed. Also there was no effect of maternal diet on metabolic changes in muscle related to high-fat diet challenge.

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국문 초록

저단백식이를 섭취한 어미의 분지아미노산 보충섭취가 비만한 자손의 근육 발달과 미토콘드리아 산화 능력에 미치는 영향

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태아 발달기간 동안의 어미의 저단백식이 섭취는 자손의 성장 및 성인기 대사성 발병 위험에 영향을 준다고 알려져 있다. 분지아미노산 (branched-chain amino acid, BCAA) 중 특히 류신은 mTOR 신호전달기전을 활성화시켜 단백질 합성을 촉진하는 역할을 한다. 어미가 저단백식이를 섭취하였을 때 주요하게 관찰되는 변화는 혈중 분지아미노산 (branched-chain amino acid, BCAA) 농도의 감소와 이로 인한 태반 및 유선발달의 저해이며 결과적으로 자손에게 전달되는 영양소가 감소된다. 이는 저단백식이를 섭취한 어미에게 BCAA를 보충하는 것이 자손의 성장 저해를 회복시킬 수 있음을 보여준다. 이러한 성장 회복은 근섬유 발달 변화를 통해 근육의 산화 능력에 영향을 줄 수 있으며 결과적으로 고지방식이에 대한 대응력을 변화시킬 수 있다. 따라서 본 연구에서는 저단백식이를 섭취한 어미에게 BCAA를 보충하는

것이 고지방식이를 섭취한 자손의 근육 성장과 산화 능력에 어떤 영향을 미치는지를 확인하고자 하였다. 암컷 ICR mice에게 임신 전 2주와 임신기 및 수유기 동안 정상단백질 식이 (20% casein), 저단백식이 (10% casein), 저단백식이에 2% BCAA를 보충한 식이 또는 2% alanine (Ala)을 보충한 식이를 공급하였다. 이때 Ala군은 BCAA 공급 군의 질소 대조군으로 사용되었다. 수컷 마우스는 이유 후부터 분석 시점 (postnatal day 175)까지 고지방 식이 (45% kcal from fat)를 공급받았으며 자손의 혈액, 비장근 (Gastrocnemius muscle) 및 넙치근 (Soleus muscle)을 분석에 사용하였다. 비장근의 무게는 NP군 대비 LP군에서 대비 유의적으로 감소하였으며 BCAA와 Ala 보충에 의해 증가하였다. 근육의 위성세포 지표인 PAX7의 유전자 발현 역시 유사한 경향으로 변화함을 확인하였다. 또한 LP군에서 유의적으로 감소한 리보솜 생합성의 지표인 S6 단백질 발현이 BCAA와 Ala 보충군에서 유의적으로 증가함이 확인되었다. 자손의 전신 인슐린 저항성은 NP군 대비 LP군에서 유의적으로 감소하였으며 BCAA 보충에 의한 변화는 관찰되지 않았다. 관찰된 인슐린 저항성의 변화와 관련하여 근육의 미토콘드리아 산화 능력을 알아보기 위해 관련 유전자의 발현을 측정하였으나 모든 지표에서 유의적인 차이가 관찰되지 않았다. 결론적으로, 어미에게 BCAA를 보충하는 것이 저단백식이 섭취로 인한 자손의 근육 성장저해에 긍정적인 영향을 미치는 것을 확인하였다. 그러나 이는 Ala 보충군에서도 관찰된 변화로 BCAA 특이적인 효과가 아닌 아미노산 보충에 의한 질소 증가로 인한 효과라고 사료된다.

또한 어미의 저단백식이 섭취로 인해 자손의 전신 인슐린 저항성이 감소하였으나 근육의 산화 능력과 관련한 변화는 관찰되지 않았다. 이는 근육이 아닌 다른 조직에서의 대사 변화가 인슐린 저항성의 변화를 야기했음을 시사한다.

주요어: BCAA, 저단백 식이, 자손 쥐, 이유 후 고지방 식이, 근육, 미토콘드리아

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