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Branched-chain Amino Acids Reverse the Growth of Intrauterine Growth Retardation Rats in a Malnutrition Model

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ABSTRACT : This experiment was conducted to determine the effect of dietary supplementation with BCAA (branched-chain amino acids: leucine, isoleucine and valine) on improving the growth of rats in a malnutritional IUGR (Intrauterine Growth Retardation) model, which was established by feeding restriction. In the experimental treatment, rats were fed purified diets supplemented with BCAA (mixed) during the whole gestation period, while arginine and alanine supplementation were set as the positive and negative control group, respectively. The results showed that, compared to the effect of alanine, BCAA reversed IUGR by increasing the fetus weights by 18.4% and placental weights by 18.0% while fetal numbers were statistically increased. Analysis of gene and protein expression revealed that BCAA treatment increased embryonic liver IGF-I expression; the uterus expressed higher levels of estrogen receptor-α (ER-α) and progesterone receptor (PR), and the placenta expressed higher levels of IGF-II. Amino acid analysis of dam plasma revealed that BCAA supplementation effectively enhanced the plasma BCAA levels caused by the feed restriction. BCAA also enhanced the embryonic liver gluconeogenesis by augmenting the expression of two key enzymes, namely fructose-1,6-biphosphatase (FBP) and phosphoenolpyruvate carboxykinase (PEPCK). In conclusion, supplementation of BCAA increased litter size, embryonic weight and litter embryonic weight by improving the dam uterus and placental functions as well as increasing gluconeogenesis in the embryonic liver, which further provided energy to enhance the embryonic growth. (**Key Words :** SD Rat, Branched-chain Amino Acids, Intrauterine Growth Retardation, Embryo, Malnutrition)

INTRODUCTION

Intrauterine Growth Retardation (IUGR) refers to the impaired growth and development of the mammalian fetus during pregnancy, which is impacted by multiple genetic, epigenetic, and environmental factors and maternal status (Wu et al., 2006). Both livestock and small mammals are found with IUGR under natural and experimental conditions (Langley and Jackson, 1994). Despite improvement of management techniques, IUGR remains a significant problem in animal agriculture because of reduced neonatal survival and a permanent stunting effect on fetal tissues and postnatal growth.

Maternal nutritional status can significantly affect intestinal absorption, the supply of amniotic and allantoic fluid nutrients, as well as the maintenance of uterine and placental function. Inadequate maternal nutrition is one of the major factors accounting for impaired fetal growth (Pennington et al., 2001; Kimball and Jefferson, 2006). Previous reports have demonstrated that protein restriction significantly decreases blood BCAA levels (Rees et al., 1999; Parimi et al., 2004). Thus, supplementation of amino acids is a potential solution to improve the maternal status and reverse restricted fetal growth.

Branched-chain amino acids (BCAA) comprise three amino acids, valine, isoleucine and leucine, which are essential amino acids for animals and the metabolism of BCAA mainly occurs in the skeletal muscle and lactating mammary gland rather than the common metabolic tissue such as liver (Finch et al., 2004; Dunshea et al., 2005). Isoleucine can be metabolized to succinyl-CoA and acetyl-CoA, both of which are important intermediates in energy metabolism, whereas valine can be metabolized to succinyl-CoA. This indicates that both isoleucine and valine can provide energy after being oxidized. Infusion of leucine or BCAA keto-acid analogues into fasting patients can improve nitrogen balance, which suggests that BCAA functions in sparing body protein (Nissen et al., 1996; Moser et al., 2000). However, the effects of BCAA supplement on fetal growth during pregnancy are still unclear.

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We thus hypothesized that BCAA supplementation during gestation may improve fetal growth and reverse IUGR by providing energy to enhance relative tissue functions. Arginine was set as a standard for evaluating the efficiency of amino acids in reversing IUGR because in a combined treatment of routine therapy, arginine significantly improved birth weight (Xiao and Li, 2005). Dietary arginine supplementation has also been reported to improve litter size and litter birth weight in gilts (Mateo et al., 2007) and rats (Zeng et al., 2008). For the isonitrogenous control, we chose alanine from the nonessential amino acids since it is considered ineffective and not toxic (Kohli et al., 2004; Wang et al., 2008). The present study was conducted to test the hypothesis in feeding controlled pregnant rats.

MATERIALS AND METHODS

Rats and diet

A total of 90 virgin, female Sprague-Dawley rats, weighing 250 to 300 g, were purchased from Beijing Laboratory Animal Center (Beijing, China) and were individually housed in a temperature- and light-controlled room held at 23°C and a lighting schedule of 12 h light/12 h dark. Animal handling and procedures were reviewed and approved by the Guidelines of China Department of Agriculture and the China Agricultural University Animal Care and Use Committee.

After 3 d of acclimatization and 4 d of feed intake measurement, rats with abnormal estrous cycle selected by determination of daily vaginal smears were excluded from the study. Pregnancy was induced by overnight caging of a proestrus female with a male of proven fertility. The presence of a vaginal plug or spermatozoa in the vaginal smear on the next day was termed as day 1 of pregnancy. From this pool of pregnant rats, 20 were selected for the first experiment (model establishment) and 50 were selected for the second experiment (amino acid treatment). The initial dam body weights were similar in all the experiments (277±12 g).

In the first experiment, a malnutrition rat model was established by restricting feed intake to 30% of normal intake for the whole gestation period. At delivery, the pups body weights and litter size were recorded to evaluate whether the model was successfully established or not (Woodall et al., 1999). In the second treatment experiment, BCAA effects on reversing IUGR were evaluated by supplementing BCAA in the diet, and the arginine- and alanine-supplemented diets were the positive and negative controls respectively. Pregnant rats were thus assigned randomly to 5 different treatments with 10 dams per treatment, including 1 full-fed group, 1 restricted-fed group, and 3 groups receiving supplements as malnutrition groups i) BCAA (44.0% leucine, 25.5% isoleucine and 30.5% valine; this amino acid ratio was in accordance with the proportion of BCAA requirement in 1995 NRC *Rat*/Mouse), ii) arginine and iii) alanine (Table 1). The supplementation of the corresponding amino acids was prepared in an

Table 1. Formulation of the diets and feed intake

Ingredient (%)	Full DI[1]	Restricted DI[1]	Alanine[2]	Arginine[2]	BCAA[12]
Cornstarch	53.00	53.00	50.96	52.00	50.11
Methionine	0.30	0.30	0.30	0.30	0.30
Casein	20.00	20.00	20.00	20.00	20.00
Soybean oil	7.00	7.00	7.00	7.00	7.00
Sucrose	10.00	10.00	10.00	10.00	10.00
Fiber	5.00	5.00	5.00	5.00	5.00
Vitamin mix[3]	1.00	1.00	1.00	1.00	1.00
Mineral mix[4]	3.50	3.50	3.50	3.50	3.50
Choline	0.20	0.20	0.20	0.20	0.20
BCAA					2.90
L-arginine				1.00	
L-alanine	1.00	1.00	2.04	1.00	1.00
Feed intake[5]	100	30	30	30	30

[1] BCAA = Branched-chain amino acids, DI = Dry matter intake.

[2] BCAA = Arginine and alanine diets are isonitrogenous.

[3] Vitamin mix provided per kilogram of diet: 0.08 mg of vitamin A, 0.33 mg of vitamin D3, 66.7 mg of vitamin E, 1.2 mg of vitamin K, 3.4 mg of vitamin B_1, 5.0 mg of vitamin B_2, 4.1 mg of vitamin B_6, 10.2 mg of nicotinic acid, 20.4 mg of pantothenic acid, 6.7 mg of biotin, 1.4 mg of folic acid, 0.02 mg of vitamin B_{12}.

[4] Mineral mix provided per kilogram of diet: 0.27 g of Mg (as MgO), 0.78 g of Ca (as $CaCO_3$), 5.36 g of P (as $CaHPO_4$), 55 mg of Mn (as $MnSO_4 \cdot H_2O$), 50 mg of Zn (as $ZnSO_4 \cdot H_2O$), 80 mg of Fe (as $FeSO_4 \cdot H_2O$), 5 mg of Cu (as $CuSO_4 \cdot 5H_2O$), 0.1 mg of Se (as Na_2SeO_3), 0.28 mg of I (as $Ca(IO_3)_2$), 0.10 mg of Co (as $CoCl \cdot 6H_2O$).

[5] The percentage of restricted group DI in full group DI (g:g). Mean weight of Full DI: 28.75 g; means of restricted DI, BCAA, arginine, alanine are 8.43 g, 8.54 g, 8.36 g and 8.36 g respectively.

isonitrogenous manner, based on a cornstarch-, casein-, soy bean oil- and sucrose-based purified rodent diet (Table 1). The feed manufacturing, including mixing and grinding, was finished in the feed workshop of the laboratory. The rats of the full intake group were fed *ad libitum* while the other 4 groups were fed 30% of their normal intake. Rats were allowed to consume water *ad libitum* during the gestation period. On day 20, all rats were anesthetized with sodium barbital and blood samples were taken from the abdominal aorta. The blood samples were centrifuged at 3,500×g for 10 min (Ciji 800 Model Centrifuge, Surgical Instrument Factory) and the sera were stored at -20°C until analysis. Litter size, placenta weight, and embryonic pup weights were recorded. The tissues of dam uterus and placenta, and embryonic liver were immediately frozen in liquid nitrogen for further analysis.

Total RNA extraction and real-time PCR

Total RNA was isolated from uterus, placenta and fetal liver using the RNAqueous Kit according to the manufacturer's instruction (Amibion, California, USA). RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1.1% denaturing agarose gel in the presence of 2.2 M formaldehyde. Total RNA was reverse transcribed to synthesize single strand cDNA. RT reaction was subjected to PCR in order to amplify ER-α, PR, IGF-I, IGF-II, FBP and PEPCK. β-actin gene was set as the internal control. The primers are listed in Table 2. The PCR system consisted of 5.0 μl of SYBR Green qPCR mix, 1.0 μl of cDNA, 3.6 μl of double distilled water, and 0.4 μl of primer pairs (25 mmol/L forward and 25 mmol/L reverse) in a total volume of 10 μl. Negative controls without RNA or with non-reverse transcribed RNA were included in all the experiments. PCR was performed using standard protocols using the annealing temperatures listed in Table 2, and then extended for 45 s at 72°C. A final extension cycle was performed at 72°C for 10 min. The melting curve program was 65-95°C, with a heating rate of 0.1°C/s and continuous fluorescence measurement. All samples were measured in triplicate. The relative mRNA levels of target genes were determined using the relative standard curve method (Zhou et al., 2007). All PCR products were sequenced to verify the specific gene products.

Western blot analysis

Tissues were pre-weighed and homogenized in ice-cold buffer (20 mM Tris; 0.1 mM EDTA; 0.1% triton-X; 250 mM sucrose; and 50 μl/5 ml protease inhibitor mixture). After centrifugation at 10,000×g for 30 min, protein concentration was then determined, tissues (50 μg) were diluted in Laemmli sample buffer (Laemmli, 1970) and resolved on a 10% (w/v) SDS-polyacrylamide gel by means of the Bio-Rad Mini-PROTEAN III Cell System. The proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham). The blot was blocked overnight at 4°C in TRIS-buffered saline (TBS) containing 5% (w/v) non-fat dried milk and incubated for 1 h at room temperature in TBS and 0.5% nonfat dry milk containing 0.2 μg/ml primary antibodies. The membrane was then washed twice at room temperature for 15 min in TBS and incubated for 1 h at room temperature in TBS containing 0.5% non-fat dried milk, with a 1:2,000 dilution of secondary antibody conjugated

Table 2. Primer sequences used for real-time PCR and primary antibodies used for Western Blot

Items	Primers (5'-3')	Fragment length (bp)	Annealing temperature (°C)	Genbank access No.	Primary antibody
β- actin	F: AACACCCCAGCCATGTACG	254	62	EF156276.1	sc-1616
	R: ATGTCACGCACGATTTCCC				
ER-α[1]	F: AATTCTGACAATCGACGCCA	344	62	NM_012689	sc-542
	R: GTGCTTCAACATTCTCCCTCCTC				
PR[1]	F: CTCCTGGATGAGCCTGATG	293	60	NM_022847	sc-538
	R: CCCGAATATAGCTTGACCTC				
IGF-I	F: ATCTCTTCTACCTGGCACTCTG	192	61	NM_001082477	sc-7144
	R: GAAGCAACACTCATCCACAAT				
IGF-II	F: TCTCTGAACGCTTCGAGCTC	300	59	NM_031511	sc-5622
	R: GAGGCTGCTTCCCGCAGCTG				
FBP[1]	F: CACCTGCCTGCACCTTTAGC	608	58	NM_012558	sc-11101
	R: CAGTTGACGCCACAATTCA				
PEPCK[1]	F: GCAGCATGGGGTGTTTGTAGG	666	64	NM_198780	sc-32879
	R: TCCCTAGCCTGTTCTCTGTGC				

[1] ER-α = Estrogen receptor-α, PR = Progesterone receptor, IGF = Insulin-like growth factor, FBP = Fructose-1, 6-biphosphatase, PEPCK = Phosphoenolpyruvate carboxykinase.

Table 3. Pup weight in the established IUGR rat model

Items	Restricted group[1]	Control group
Pup weight (g)	5.24±0.07	6.46±0.09**[2]
Litter size (n)	10.2±0.89	12.1±0.97**
Litter embryonic pup weight (g)	53.45±0.51	78.17±0.64**
Litter placenta weight (g)	13.97±0.09	17.42±0.11**

[1] The percentage of restricted group DI is 30% of full DI: 30%. DI: dry matter intake.
[2] Difference from the restricted group, ** $p<0.01$.

with horseradish peroxidase (Amersham). The immune complex was detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce). All primary antibodies were purchased from Santa Cruz (California, USA) and information on primary antibodies is provided in Table 2.

Analytical methods

Serum glucose was determined with the Chemistry Analyzer (RA1000, Bayer Corporation, Germany) and Glucose Reagent kit (Biosino Bio-technology and Science Inc., Beijing, China). Serum insulin was measured with the Rat Insulin ELISA kit (Rapidbio, California, USA) according to the manufacturer's instruction. The concentration of amino acids in maternal plasma was measured using an S-433D amino acid analyzer (SYKAM) according to the Ninhydrin post-column derivatization method using amino acid standards procured from Sigma.

Statistical analysis

One-way ANOVA followed by a Tukey multiple comparison test was performed to determine differences in plasma amino acids and tissue gene expression. Data were analyzed using the procedures of SAS (SAS Institute). Results are expressed as means±SEM. $p<0.05$ was considered significant.

RESULTS

The establishment of a feed restricted IUGR model

A full dry matter intake was first evaluated by feeding dams with purified diets *ad libitum*, and the actual feed intake was recorded daily for five days. The ratio of actual feed intake to dam body weight (g/g) was calculated as the mean of the five days intake (g) divided by the dam body weight (g). After being weighed individually, all rats were then randomly assigned into two groups, the fully fed group and the feed restriction group. Thirty percent feed restriction, which was adjusted according to dam body weight, was initiated once the dam was confirmed pregnant (Woodall et al., 1999). At parturition, both the restriction- and full-fed rats were allowed to deliver the pups, which were weighed. The pup body weights of the restriction group were all decreased significantly, and lower by three times the standard error of the mean normal pup body weight (Table 3). This confirmed the successful establishment of the feed restriction IUGR model.

BCAA reversed IUGR by improving the pups birth weight and total fetal weight

The experimental treatment was started on the first day of gestation. All pregnant dams, which showed no difference in initial body weight, were assigned into five treatments which were BCAA treatment, arginine treatment, alanine treatment, restricted-fed treatment (30% of full-fed), and the full-fed group respectively. Table 1 lists the actual feed intake of these five groups of rats. On gestation day 20, all dams were killed for evaluation of fetal body weight and litter size. The average embryo body weights of both BCAA and arginine treatments increased when compared with the control alanine treatment (Table 4) ($p<0.01$, $p<0.05$). The effect of BCAA was more significant (22.2%) than the

Table 4. Reproductive performance, serum glucose and insulin of diet-treated rats[1]

Item	Full DI	Restricted DI	Alanine	Arginine	BCAA[2]
Maternal DI[2] (g/d)	28.7±1.75**	8.43±0.86	8.36±0.93	8.36±0.92	8.54±0.75
Litter size (n)	12.0±0.94*	9.8±0.92	10.4±0.97	11.4±0.97*	11.4±1.07*
Embryonic pup weight (g)	2.24±0.19**	1.62±0.17	1.67±0.12	1.81±0.10*	1.98±0.23**
Litter embryonic pup weight (g)	26.74±1.24**	15.73±1.01	17.32±1.70	20.62±1.39**	22.32±1.24**
Placenta weight (g)	4.89±0.36**	3.28±0.20	3.52±0.32	4.15±0.33**	4.15±0.40**
Glucose (mg/dl)	99.2±9.1	88.8±9.16	87.7±9.99	88.3±9.8	87.3±9.33
Insulin, (IU/ml)	16.44±2.01	18.01±2.4	18.33±3.75	18.81±3.37	20.5±2.99

[1] Data are means±SEM. * Different from the Alanine group, $p<0.05$; ** $p<0.01$.
[2] DI = Dry matter intake, BCAA = Branched-chain amino acids.

Table 5. Amino acid concentrations (nmol/ml) in rat dams fed on treatment diets[1]

Diet	Full DI[2]	Restricted DI	Alanine	Arginine	BCAA[2]
Asparagine	155±17[a]	198±21[b]	184±11[b]	173±20[b]	162±5[b]
Glutamate	664±51	1121±92	927±67	1313±105	1,640±154
Histidine	174±23	157±43	169±32	160±27	164±4
Serine	290±45[a]	408±72[b]	299±39[a]	414±59[b]	384±36[ab]
Glycine	145±15[a]	210±7[b]	168±24[ab]	165±10[ab]	146±8[ab]
Threonine	408±52[a]	265±33[b]	289±42[b]	301±9[b]	249±19[b]
Alanine	926±91	1109±87	894±74	828±65	1010±90
Arginine	296±33[b]	287±5[b]	295±12[b]	391±25[a]	303±19[b]
Valine	239±17[a]	161±9[b]	203±19[ab]	199±27[ab]	249±33[a]
Isoleucine	140±8[a]	94±11[b]	126±7[ab]	110±20[a]	152±23[a]
Leucine	179±5[a]	101±14[b]	123±10[b]	141±31[ab]	198±6[a]
Methionine	81±8	87±10	80±13	61±9	76±23
Tyrosine	144±32	137± 22	157±10	111±33	124±16
Phenylanine	142±21	157±33	183±8	166±27	149±52
Tryptophan	89±38	84±25	73±17	81±20	69±7
Cysteine	254±22	276±14	289±40	266±29	213±31
Lysine	912±59	801±77	904±61	773±18	916±40

[1] Values were expressed as mean±SEM. [a, b, c] Mean values within a row not sharing a common superscript letter are significantly different, $p<0.05$ (ANOVA).
[2] DI = Dry matter intake, BCAA = Branched-chain amino acids.

arginine treatment (11.7%) compared to the restricted-fed group. Both BCAA and arginine tended to increase litter size compared to alanine treatment ($p<0.05$). Notably, the total fetal weight of BCAA was increased by 28.9% ($p<0.01$), which was higher than that of arginine treatment (8.2% increase) ($p>0.05$). Placental weights were increased significantly in the BCAA and arginine treatments ($p<0.01$), and the effects were similar.

Supplementation of BCAA reverses plasma BCAA levels in the feed restriction

Previous reports have demonstrated that protein restriction significantly decreases blood BCAA levels (Rees et al., 1999; Parimi et al., 2004). In this study, we thus measured the dam plasma amino acid concentration (Table 5). BCAA (valine, isoleucine and leucine) supplementation effectively increased BCAA levels in plasma compared to the alanine and arginine treatments. Plasma serine, asparagine and glycine were increased while plasma threonine was decreased in the restricted-fed rats. BCAA supplementation did not significantly increase these amino acids in the plasma.

BCAA improved the uterine and placental functioning

To further reveal the underlying mechanism, we examined gene expression pattern and protein expression in dam and embryo tissues. The uterine ER$\tilde{}$ was significantly enhanced by BCAA treatment (94%) (Figure 1a), and arginine increased its expression by 86%, which demonstrated that BCAA functioned more profoundly than arginine ($p<0.01$) on regulation of ER-α expression. Uterine PR expression was also augmented by BCAA and arginine treatments (35%, 36%) with no difference between the two treatments ($p>0.05$) (Figure 1b) in both gene and protein expression.

Both BCAA and arginine enhanced the transportation of placenta to a similar level (98.6%, 97.6%), which was demonstrated by placental IGF-II expression (Figure 1c). These observations indicated that placental function can be improved by BCAA and arginine to a similar degree, and the different enhancing effect on embryonic growth is beyond the placenta transportation.

BCAA promoted IGF-I expression in fetal liver by 50.4%, while arginine increased it by 25.6%, both of which were significantly different from the restricted-fed group ($p<0.05$) (Figure 1d). The difference between BCAA and arginine groups was also significant ($p<0.05$). This result probably can explain the difference in fetal weights of BCAA and arginine treated animals. It also implies a distinct functional mechanism of BCAA and arginine in reversing IUGR in this malnutrition model.

BCAA enhanced embryonic liver gluconeogenesis but not in dams

To further reveal the functional mechanism, we examined dam blood glucose and insulin levels in order to identify the BCAA effect on dams. The results (Table 4) demonstrated that neither blood glucose or insulin levels

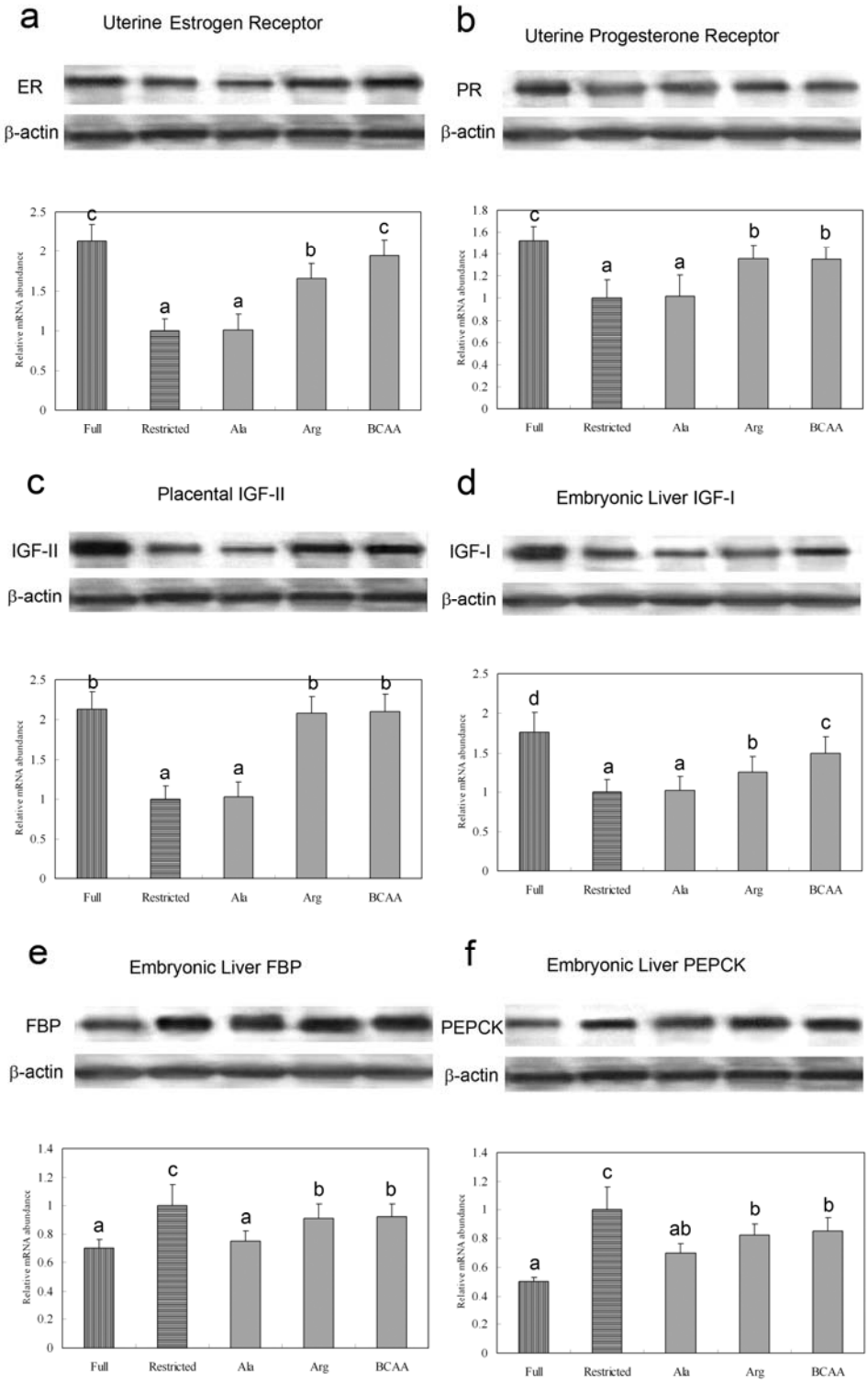

Figure 1. Western blot analysis and gene quantification of uterine hormonal receptors (ER, PR), Placental IGF-II, embryonic liver IGF-I, FBP and PEPCK in diet treated rats. Dam uterine estrogen receptors (a) and progesterone receptors (b); placental IGF-II (c); embryonic liver IGF-I (d), FBP (e) and PEPCK (f). The five diet treatments included full feeding (Full), restricted feeding (Restricted), alanine supplement (Ala), arginine supplement (Arg) and BCAA supplement (BCAA). Relative mRNA abundance is expressed as means±SEM in the bar graph. Values not sharing a common letter were significantly different, $p<0.05$ (ANOVA). BCAA: branched-chain amino acids, ER-α: estrogen receptor-α, PR: progesterone receptor, IGF: insulin-like growth factor, FBP: fructose-1, 6-biphosphatase, PEPCK: phosphoenolpyruvate carboxykinase.

differed among all amino acid treatments, even between the restricted-fed and full-fed groups. This indicated that under a malnutrition condition, the body can shift precursors from other tissues to maintain blood glucose. Hence, feed restriction in the experiment did not alter dam blood glucose. We then quantified the gene expression of two key enzymes of gluconeogenesis, fructose-1, 6-biphosphatase (FBP) and phosphoenolpyruvate carboxykinase (PEPCK), to study whether BCAA affected liver gluconeogenesis in both dams and embryos. The dam genes of FBP and PEPCK were not significantly affected by treatments (data not shown), whereas the embryonic FBP and PEPCK were significantly augmented in the BCAA and arginine groups (Figure 1e, 1f). The expression of these two genes was enhanced to a similar degree by BCAA (22.7%, 21.4%) and arginine (20.0%, 17.4%) compared to the alanine treatment. When compared with the full-fed group, all restricted-fed groups displayed higher expression of FBP and PEPCK in embryonic liver. This implied that in a malnutrition condition, embryonic liver gluconeogenesis is simultaneously induced to provide energy for growth.

DISCUSSION

Maternal nutritional status is one of the most important factors for IUGR (Pennington et al., 2001). Different animals show variable abilities to tolerate shifts in maternal nutrition. Sheep, heifers and mares have a poor capacity to tolerate maternal malnutrition, which always causes low fetal birth weight, uterine contractibility, and increased perinatal mortality, and is associated with fetal hypoglycemia and hypoxemia etc (Jozwik et al., 1999). Pigs generally can tolerate maternal malnutrition during fetal growth. Even a 50% reduction in energy intake will not affect piglet birth weight (Atinmo et al., 1974). During animal production, maternal or fetal undernutrition occurs frequently, especially in grazing animals. Previous study has reported a restricted *ad libitum* model in Wistar rats (Woodall et al., 1996). In this study, we firstly established a Sprague-Dawley rat IUGR model by 30% restriction of normal feed intake during the gestation period for investigation of nutritional reversing on malnutritional IUGR.

In clinical trials, attempts have been made to prenatally treat IUGR with zinc (George et al., 1998; Roungsipragarn et al., 1999), fish oil (Olsen et al., 2000; Olsen et al., 2007) and oxygen therapy (Almström and Sonesson, 1996; Brantberg and Sonesson, 1999). Hormones such as IGF-I and growth hormone showed little effect in malnutrition of rats (Woodall et al., 1999), but amniotic IGF-I administration reversed IUGR in normal sheep (Eremia et al., 2007). In animal production, these therapies appear impractical due to high cost. The IUGR abandonment of offspring causes great economic loss for farms annually. Under restricted protein feeding, blood BCAA levels were significantly decreased (Rees et al., 1999; Parimi et al., 2004). Whether nutritional supplements can relieve the malnutritional IUGR is becoming a research focus. Therefore, knowledge about the nutrients regulating fetal growth could be vital for this field of research.

The three amino acids (leucine, isoleucine and valine) of BCAA share similar structure, membrane transporters and metabolic pathways (Holecek, 2002; Finch et al., 2004; Dunshea et al., 2005). Valine, leucine and isoleucine appear to be essential for the lactating pig. In the mammary gland, isoleucine and valine are metabolized to provide energy (Moser et al., 2000). BCAA are hormone secretagogues as well. Leucine and isoleucine can stimulate insulin and inhibit glucagon secretion simultaneously (Nair and Short, 2005). In this study, we showed that BCAA improved embryonic weights by enhancing dam uterus function as indicated by upregulated ER-α expression, which increases sensitivity of the uterus to hormones in under-nutrition conditions. The uterus is the tissue which prepares a bed for fertilized ova, nourishes the developing embryo during pregnancy, and expels the fetus. Progesterone and estrogen play central roles in the estrous cycle and the maintenance of pregnancy. The corresponding progesterone and estrogen receptors are regulated in response to these hormones during the maintenance of physiological status (Fitzgerald et al., 2001). In the present study, we tested the estrogen and progesterone receptors in the uterus in order to reveal the uterine sensitivity to circulating hormones under the treatment. Increased ER-α expression improved uterus functioning and remodeling during gestation by responding to the circulating estrogen. Compared to the significant augmentation of ER-α expression (91%), the impact of BCAA on the expression of PR was less enhanced (35%). Even the fully-fed dam uterus displayed only moderately increased PR expression (52%). These results indicate that ER-α might be more sensitive to energy supplies than PR, which is reasonable considering the receptor function. PR serves a maintenance role in gestation, while ER-α is correlated to the uterus functional structure. The placental function was also enhanced as demonstrated by the marker protein IGF-II expression (Constância et al., 2002). This implies that BCAA enhanced the dam function via an unknown pathway, which increased embryonic growth by augmenting liver IGF-I expression. The BCAA effect was more significant in the uterus than in the placenta and fetal liver, which indicates the first target of BCAA on the dam uterus rather than the placenta-embryonic transportation. The regulation mechanism of BCAA on these genes is still unknown. One possible mechanism is to regulate gene expression at the translation level. BCAA, especially leucine, are one of the translation initiation factors in

muscle (Bolster et al., 2004; Escobar et al., 2005), which suggests a possible regulation mechanism in other tissues.

Metabolism of BCAA differs from most of the other essential amino acids. They cannot be metabolized into unique biologically active molecules (Brosnan and Brosnan, 2006), which might be one of the reasons that the mechanism by which BCAA functions is still unclear. Studies have been focused on their metabolism which shows that BCAA are firstly reversibly catabolized to their respective α-keto acids by branched-chain amino acid aminotransferase (BCAT) and then irreversibly oxidatively decarboxylated into acyl-COA derivatives by a mitochondrial branched-chain α-keto acid dehydrogenase (BCKD) complex (Harper et al., 1984). There are two BCAT isoenzymes including mitochondrial BCAT (BCATm) and cytosol BCAT (BCATc) (Suryawan et al., 1998). In rats, BCATm is ubiquitously distributed whereas BCATc is restricted to brain, ovary and placenta (Daeipour et al., 1993). BCKD can be detected in all BCATm expressed tissues. In this study, we found that BCAA supplementation in diets did not change dam plasma insulin but enhanced the expression of placental IGF-II and embryonic liver IGF-I. Whether BCAA could be catabolized in the placenta or transferred via placenta to the embryo to function needs further investigation. However, embryonic liver gluconeogenesis was enhanced by increased expression of FBP and PEPCK. This can be explained partially by an intermediate in the valine catabolism pathway, β-hydroxyisobutyrate, which is a gluconeogenic substrate. Similarly, whether BCAA or their metabolites can pass the placenta to enhance embryonic liver gluconeogenesis also needs to be studied.

Though arginine has been indicated to be effective in reversing IUGR by improving embryonic survival and increasing placental-embryonic blood flow (Xiao and Li, 2005; Mateo et al., 2007; Zeng et al., 2008), the effect of arginine was not as pronounced as BCAA in this study, which indicates that BCAA performs more effectively in an under-nutrition model. This might function via an energy improvement rather than blood flow alteration.

CONCLUSION

In conclusion, supplementation of branched-chain amino acids to the diet for female rats throughout the gestation period increases litter size, embryonic weight and litter embryo weight by improving the uterus and placental functions of dams as well as increasing gluconeogenesis of BCAA in embryonic liver, which provides energy to enhance fetal growth. Our novel findings have important implications for reversing intrauterine growth retardation and improving pregnancy outcome in mammals.

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