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"Dietary supplementation of L-tryptophan" increases muscle development, adipose tissue catabolism and fatty acid transportation in the muscles of Hanwoo steers

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Abstract

This study investigated the effects of dietary rumen-protected L-tryptophan (TRP) supplementation (43.4 mg of L-tryptophan kg^{-1} body weigt [BW]) for 65 days in Hanwoo steers on muscle development related to gene expressions and adipose tissue catabolism and fatty acid transportation in *longissimus dorsi* muscles. Eight Hanwoo steers (initial BW = 424.6 kg [SD 42.3]; 477 days old [SD 4.8]) were randomly allocated to two groups (n = 4) of control and treatment and were supplied with total mixed ration (TMR). The treatment group was fed with 15 g of rumen-protected TRP (0.1% of TMR as-fed basis equal to 43.4 mg of TRP kg^{-1} BW) once a day at 0800 h as top-dressed to TMR. Blood samples were collected 3 times, at 0, 5, and 10 weeks of the experiment, for assessment of hematological and biochemical parameters. For gene study, the *longissimus dorsi* muscle samples (12 to 13 ribs, approximately 2 g) were collected from each individual by biopsy at end of the study (10 weeks). Growth performance parameters including final BW, average daily gain, and gain to feed ratio, were not different ($p > 0.05$) between the two groups. Hematological parameters including granulocyte, lymphocyte, monocyte, platelet, red blood cell, hematocrit, and white blood cell showed no difference ($p > 0.05$) between the two groups except for hemoglobin ($p = 0.025$), which was higher in the treatment than in the control group. Serum biochemical parameters including total protein, albumin, globulin, blood urea nitrogen, creatinine phosphokinase, glucose, nonesterified fatty acids, and triglyceride also showed no differences between the two groups ($p > 0.05$). Gene expression related to muscle development (Myogenic factor 6 [*MYF6*], myogenine [*MyoG*]), adipose tissue catabolism (lipoprotein lipase [*LPL*]), and fatty acid transformation indicator (fatty acid binding protein 4 [*FABP4*]) were increased in the treatment group compared to the control group ($p < 0.05$). Collectively, supplementation of TRP (65 days in this study) promotes muscle development and increases the ability of the animals to catab-

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Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Priatno W, Jo YH.
Data curation: Priatno W, Jo YH.
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All experimental procedures were in accordance with the "Guidelines for Care and Use of Experimental Animals" of Konkuk University (Approval no: KU18178).

olize and transport fat in muscles due to an increase in expressions of *MYF6*, *MyoG*, *FABP4*, and *LPL* gene.

Keywords: Adipose tissue catabolism, Fatty acid transportation, Growth performance, Hanwoo steers, Muscle development, Rumen-protected L-tryptophan

INTRODUCTION

L-Tryptophan (TRP) is an essential amino acid (AA) which requires to be supplemented in diets to meet the maximum production quality [1–3]. It is while most AAs are commonly assumed not to be deficient in cattle. Ruminal fermentation produces the microbial protein, which is supplied to the small intestines and delivers a good quality pattern of both essential and non-essential AAs, about 34%–89% of total protein [4,5]. The amounts of essential AAs supplied by microbes are sufficient to support maintenance and normal physiological status, but not maximal growth, milk, and beef production [6–8]. Although the assumption is that cows produce sufficient AA through their diet; however, numerous common feed ingredients (average CP = 23% of dry matter [DM], minimum CP = 3%, maximum CP = 95.5% of DM basis) contain an average concentration of TRP at approximately 1% or less of total CP [5]. Therefore, the proteins supplied from the diet do not contain sufficient essential AAs to meet the animal's needs. In addition, normal protein supply could not be used efficiently for muscle synthesis [9]. This experiment aimed to investigate the effect of additional TRP as an essential amino acid (block) for bundling protein of muscles as its amount is lacking when maximum production is targeted. Thus, we hypothesized that TRP supplementation may improve muscle development, and thus worth investigation in the present study.

Previous literature investigated the relationship between reducing stress and TRP addition using injection or infusion to horses [10], dairy cows [11], and beef cattle [2]. However, there is a paucity of information regarding its supplementation effect on *longissimus dorsi* muscle and the related gene expression in Korean beef cattle. Thus, we hypothesized that TRP might not only be a building block for muscle development in steers but also induce gene-expression related muscle development and adipose tissue catabolism and fatty acid transportation in *longissimus dorsi* muscle tissue.

MATERIALS AND METHODS

Animals and management

All experimental procedures were in accordance with the "Guidelines for Care and Use of Experimental Animals" of Konkuk University (Approval no: KU18178). Eight Hanwoo steers (initial body weigt [BW] = 424.6 kg [SD 42.3]; 477 days old [SD 4.8]; Castration age 189 days old [SD 4.8]) were randomly allocated to two groups of control and treatment (n = 4) and supplied with diets with the total mixed ration (TMR; Table 1). The 477 days old steers have muscle and intramuscular fat in *longissimus dorsi* muscle. Thus, we selected the period for analyzing adipose tissue and muscle metabolism in *longissimus dorsi* muscle. The treatment group was fed 15 g of rumen-protected TRP (0.1% of TMR as-fed basis equal to 43.4 mg of TRP kg^{-1} BW; CJ CheilJedang, Suwon, Korea) provided as top dressing once a day at 0800 h. The rumen-protected TRP was conjugated of acetate and TRP. The amount added was triple times higher than the concentration provided in the control diet (Table 1). The rate of by-pass was indicated above 95% [3]. TRP was included at 0.829 g per 1 g of rumen-protected TRP. The treatment group was supplied by oral administration for each steer for 10 weeks.

The animals were placed in 10 m^2 (2 m [length] × 5 m [breadth]) with two steers per pen at Konkuk University farm (Chungju, Korea). All animals in the control and treatment groups were

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Table 1. Composition and nutrient content of experimental diets for steer (DM basis)

Item	Concentration	
	Basal diet	Dietary supplementation of rumen-protected TRP
Chemical composition (%)		
Moisture	42.7	
Crude protein	18.5	
Ether extract	5.3	
Crude fiber	15.1	
Neutral detergent fiber	30.4	
Acid detergent fiber	18.1	
Ca	1.2	
P	0.6	
ME (Mcal/kg)	2.7	
NE_m (Mcal/kg)	1.8	
NEg (Mcal/kg)	1.2	
Amino acids (total intake g/day [DM basis])		
Alanine	63.7	
Arginine	31.8	
Aspartic acid	57.6	
Cystine	21.5	
Glutamic acid	128.2	
Glycine	36.1	
Histidine	18.1	
Isoleucine	25.8	
Leucine	77.4	
Lysine	31.8	
Methionine	11.2	
Phenylalanine	34.4	
Proline	68.0	
Serine	34.4	
Threonine	31.0	
Tryptophan[1)]	6.0	18.2
Tyrosine	16.3	
Valine	37.9	

[1)]Supplementation of 15 g rumen-protected TRP (as-fed basis). The institute of CJ CheilJedang supplied rumen-protected TRP, TRP were included 0.829 g per 1 g of rumen-protected TRP.

DM, dry matter; TRP, L-tryptophan.

fed 15 kg of TMR feedstuff (as-fed basis) once a day at 0800 h. Water was available *ad libitum*. Residues of TMR were recorded for each pen every day prior to providing the fresh TMR and water. Then, we used the equation from pen data to individual data. Dry matter intake (DMI) equation was calculated as follows: DMI/BW % = 1.2425 + 1.9218 × net energy for maintenance (NE_m) – 0.7259 × $(NE_m)^2$ [12].

Blood biochemical and hematological parameters in Hanwoo steers

Blood samples from each steer were obtained at 1400 h via jugular venipuncture into the non-heparinized vacutainers (20 mL; Becton-Dickinson, Belliver Industrial Estate, PL6 7BP, Plymouth,

UK) to obtain serum for measuring biochemical parameters and the EDTA-treated vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) to obtain whole blood for measuring hematological parameters at 0, 5, and 10 weeks. The collected blood samples were stored on ice before transferring to the laboratory. Then, blood in serum tubes was centrifuged at 2,740×g for 15 min at 4℃. The serum was transferred to a 1.5 mL tube and stored in a freezer (−80℃) for further analysis.

Protein metabolism was evaluated by measuring serum levels of total protein (TP), albumin (ALB), globulin (GLO), creatinine phosphokinase (CPK), and blood urea nitrogen (BUN). Energy metabolism was evaluated by measuring the serum level of glucose (GLU), triglyceride (TG), and nonesterified fatty acids (NEFA). These biomarkers were assayed by using a biochemical autoanalyzer (FUJI DRI-CHEM 7000; Fujifilm, Tokyo, Japan). Whole blood samples were used for analyzing complete blood-cell count, including granulocyte (GRA), lymphocyte (LYM), monocyte (MON), platelet (PLT), red blood cell (RBC), hematocrit (HCT), hemoglobin (HGB), and white blood cell (WBC) using VetScan HM2 (Diamond Diagnostics, Abaxix, Holliston, MA, USA).

Sampling from *longissimus dorsi* muscle and extracting total RNA

Longissimus dorsi muscle samples (12 to 13 ribs, approximately 2 g) were collected from four steers in each group via surgery performed by a professional veterinarian at the end of the study (10 weeks). Briefly, local anesthetic agents were administered at 6 equidistant points around the resection area, namely, the *longissimus dorsi* muscle at position 12 to 13 of the ribs. The veterinarian resected 5 cm of skin in the direction of the muscle and then dissected the *longissimus dorsi* muscle tissue using surgical equipment. The collected *longissimus dorsi* muscle tissues were washed immediately into DEPC-treated distilled water (diethyl pyrocarbonate, Sigma-Aldrich, St. Louis, MO, USA) for protecting them from RNase contamination. The tissues were then preserved in 50-mL tubes after dipping into liquid nitrogen and placed in dry ice until transferring to the laboratory. The tissue samples were ground into powder in liquid nitrogen. The TRIzol was used to extract total RNA to follow up the method of [13]. The concentration of RNA was detected by spectrophotometric analysis (NanoDrop 1000, Thermo Scientific, Massachusetts, MA, USA). The RNA integrity was assessed using an RNA nano 6000 assay kit for an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA , USA). When the RNA integrity number (RIN) was 6 or higher, it was used for complementary DNA (cDNA) synthesis. The RNA used for this real-time polymerase chain reaction (PCR) analysis was RIN = 6.5 (SD = 0.27).

Synthesis of cDNA and gene expression analysis

To synthesize cDNAs, 1 μg of RNA was reverse transcribed in a 100 μL reaction volume with an iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed on duplicate samples by using a CFX Connect TM Real-Time System (Bio-Rad) with IQTM SYBR Green Supermix reagents (Bio-Rad). The following PCR conditions were used: 95℃ for 3 min and 40 cycles at 95℃ for 10 s, 51℃ to 61℃ for 30 s and 72℃ for 30 s. Expression of mRNA of gene involved in muscle development (myogenic factor 6 [*MYF6*], myoblast determination [*MyoD*], and myogenie [*MyoG*]), fat accumulation (CCAAT/enhancer-binding protein alpha [*C/EBPα*] and peroxisome proliferator-activated receptor gamma [*PPARγ*]), adipose tissue catabolism (lipoprotein lipase [*LPL*], stearoyl-CoA desaturase [*SCD*]), and fatty acid transportation (fatty acid binding protein 4 [*FABP4*]). 18S ribosomal RNA (*18S*), glyceraldehyde3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein lateral stalk subunit P0 (*RPLP0*) primers were designed using the National Center for Biotechnology Information website. *18S, GAPDH*, and *RPLP0* were used as housekeeping genes [3,14]

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(Table 2).

Statistical analysis

The GLM procedure of SAS (SAS 9.4 Institute, Cary, NC, USA) was employed for two-way analysis of variance to analyze our data. Growth performance data (BW, average daily gain [ADG], and gain to feed ratio [G:F]), and mRNAs expression were assessed using Student's *t*-tests. Data of blood biochemical and hematological parameters were used for mixed procedure of SAS using repeated measures. The model included the fixed effect of diet (control and 15 g rumen protected TRP group), and the random effects of blood sampling time. Blood was collected three times (0, 5, and 10 weeks). Data were presented as least-square means. Significance was set at $p \leq 0.05$, and the tendency was declared at $0.05 < p \leq 0.1$.

RESULTS AND DISCUSSION

The amount of TRP in the treatment group was about 3 times higher than the amount in the control group. However, no differences were observed in growth performance parameters including BW, ADG, and G:F ($p > 0.05$; Table 3). Ma et al. [15] supplied 3 doses of rumen-protected TRP (48.1, 96.2, and 144.2 mg kg^{-1} BW) to cashmere goats, and found increased BW and ADG by supplementation of 96.2 and 144.2 mg to the TRP group, but no difference by supplying 48.1 mg TRP. Henry et al. [16] examined dietary supplementation of 47.9 and 83.9 mg of TRP kg^{-1} BW in comparison with the control on pigs and found higher average daily feed intake (FI), ADG, G:F, muscle gain (g/d), and fat gain (g/d) in the 83.9 mg TRP kg^{-1} BW group than in the other groups. Newborn calves that were supplied with colostrum and milk plus 1.5 mg of 5-hydroxy-TRP kg^{-1} BW during 15 days after birth showed on increased immune factors, but no changes in growth performance and blood biochemical parameters including glucose, fatty acids, calcium, and magnesium [17]. Based on the results of the previous studies, we considered that the given 43.4 mg of TRP kg^{-1} BW might not be sufficient to improve the growth parameters in the current study. Another possible explanation could be the low sample size in this study. Further study with feeding higher amounts of TRP and larger sample size may warrant the obtained results or cause different result

Table 2. Primer sequences used in quantitative reverse transcription-PCR assays

Gene symbol	Gene name	Annealing temperature (℃)	Forward primer	Reverse primer
C/EBPα	CCAAT/enhancer-binding protein alpha	60.3℃	CCGTGGACAAGAACAGCAACGA	GGCGGTCATTGTCACTGGTCAG
FABP4	Fatty acid binding protein 4	60.0℃	GTGTGATGCATTTGTAGGT	CTGGTGGCAGTGACACCAT
PPARγ	Peroxisome proliferator-activated receptor gamma	61.0℃	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG
LPL	Lipoprotein lipase	60.0℃	TACCCTGCCTGAAGTTTCCAC	CCCAGTTTCAGCCAGACTTTC
MYOD	Myoblast determination protein	59.6℃	AGAGTTGCTTTGCCAGAG	CTGCCTGCCGTATAAACA
MYF6	Myogenic factor 6	60.7℃	GAAGGAGGGACAAGCATTGA	GAGGAAATGCTGTCCACGAT
MyoG	Myogenin	59.6℃	AGAGTTGCTTTGCCAGAG	CTGCCTGCCGTATAAACA
SCD	Stearoyl-CoA desaturase	60.0℃	TCCGACCTAAGAGCCGAGAA	GCAGGATGAAGCACAACAACAG
18S	18S ribosomal RNA	51.0℃	ACCCATTCGAACGTCTGCCCTATT	TCCTTGGATTGTGGTAGCCGTTTCT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	60.0℃	GGCAAGGTCATCCCTGAG	GCAGGTCAGATCCACAACAG
RPLP0	Ribosomal protein lateral stalk subunit P0	55.0℃	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA

PCR, polymerase chain reaction.

Table 3. Growth performance of steers under rumen-protected L-tryptophan supplementation

Item	Control	Treatment	SEM	*p*-value
Initial body weight (kg)	420.5	428.8	32.1	0.806
5 weeks				
Body weight after 5 weeks (kg)	457.0	480.8	27.95	0.570
Average daily gain (kg/d)	1.04	1.49	0.25	0.263
Dry matter intake (kg/d)	7.22	7.61	0.39	0.498
Gain:Feed	0.17	0.24	0.11	0.224
10 weeks				
Body weight (kg)	493.8	510.5	27.0	0.676
Average daily gain (kg/d)	0.97	0.79	0.12	0.339
Dry matter intake (kg/d)	8.12	7.94	0.48	0.792
Gain:Feed	0.13	0.11	0.02	0.427

and thus advisable.

Hematological parameters GRA, LYM, MON, PLT, RBC, HCT, and WBC were examined and only HGB showed the higher value in the treatment group (p = 0.025; Table 4). Hemoglobin was known as an iron-containing biomolecule that transports oxygen and carbon dioxide in red blood cells [18]. Serum TRP is positively correlated with iron metabolites ($0.100 \leq r^2 \leq 0.305$) including HGB ($r^2$ = 0.301) in human. TRP is an important factor for erythropoiesis which produces red blood cells, which is the development of erythropoietic stem cells to mature red blood cells [19]. Similar with these complete blood count (CBC) results, Lee et al. [3] found no changes in WBC, LYM, GRA, RBC, HGB, HCT, mean corpuscular hemoglobin (MCH), and PLT of steers fed

Table 4. Effect of supplementation of rumen-protected L-tryptophan on the blood hematological parameters and serum biochemical parameters in Hanwoo steers

Item	Control	Treatment	SEM	*p*-value[1)]
GRA (10^3/μL)	4.06	3.39	0.75	0.450
LYM (10^3/μL)	5.59	5.85	0.79	0.742
MON (10^3/μL)	0.62	0.48	0.23	0.545
PLT (10^5/μL)	362	321	0.64	0.362
RBC (10^6/μL)	8.22	9.07	0.47	0.216
HCT (%)	33.9	36.7	1.25	0.082
HGB (g/dL)	12.4	13.7	0.43	0.025
WBC (10^3/μL)	10.3	9.8	1.10	0.732
TP (mg/dL)	6.2	6.3	0.15	0.558
ALB (g/dL)	3.3	3.5	0.11	0.224
GLO (g/dL)	3.0	2.9	0.08	0.319
BUN (mg/dL)	19.6	18.3	1.23	0.421
CPK (U/L)	161.1	218.1	68.8	0.264
GLU (mg/dL)	45.3	48.3	2.60	0.461
NEFA (mmol/L)	163.8	157.8	14.2	0.684
TG (mg/dL)	20.6	22.3	4.69	0.721

[1)]Control : n = 4, Treatment : n = 4.

GRA, granulocyte; LYM, lymphocyte; MON, monocyte; PLT, platelet; RBC, red blood cell; HCT, hematocrit; HGB, hemoglobin; WBC, white blood cell; TP, serum total protein; ALU, albumin; GLO, globulin; CPK, creatinine phosphokinase; BUN, blood urea nitrogen; GLU, glucose; NEFA, nonesterified fatty acids; TG, triglyceride.

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with 0.1% rumen-protected TRP.

All blood biochemical parameters including ALB, GLO, GLU, CPK, NEFA, BUN, TG, and TP were not changed by supplementation of TRP ($p > 0.05$; Table 4). ALB, CPK, GLO, BUN, and TP were indicators related to nitrogen utilization in blood [20]. Supplied 0.8 g TRP improved nitrogen utilization by lambs fed 2.7% non-protein nitrogen of DM [21]. Thus, we investigated effects of TRP on nitrogen utilization by using blood metabolism markers in cattle, but there was no difference between the two groups. TRP is also considered as both glucogenic and ketogenic amino acid, and thus, it is expected to see the reflected changes in some blood biochemical parameters. Additionally, since most of the factors affecting blood metabolites by supplementation of rumen-protected AAs are related to energy metabolism, protein metabolism, and DMI [22], therefore, changes in those factors could be expected due to feeding TRP in this study. However, we could not find any difference in nitrogen utilization and its index in the blood (BUN) nor in other biochemical parameters between two groups. One reason could be attributed to the similar DMI in both groups. Another possible explanation could be due to the low sample size (n = 4) and less blood sampling in this study. It should be noted that we collected blood samples prior to feeding, and thus due its half-life (1.8 h in *in-vitro* culture [23]) and also body homeostasis mechanism, rumen protected TRP may not alter blood metabolite parameters including TP, ALB, GLO, BUN, CPK, GLU, NEFA, and TG. Consistent with our results, in the previous study under cold stress conditions, Lee et al. [3] reported unchanged blood biochemical parameters by supplementation of 0.1% rumen-protected L-TRP in Hanwoo steers, when blood samples were collected prior to feeding. Further study with a larger sample size and with the addition of a higher amount of TRP supplementation may result in significant differences in blood biochemical values particularly when blood was collected after feeding.

Relative mRNAs expression including *MYF6*, *MyoG*, *FABP4*, and *LPL* were higher in the treatment group than in the control ($p < 0.05$; Fig. 1). The *MYF6* and *MyoG* are representative of muscle differentiation [24]. The *MYF6* regulates the final differentiation of myotubes and is regarded as the principal factor influencing skeletal muscle phenotype. The *MYF6* is expressed at a higher level in adult skeletal muscle than all of the other myogenic regulatory factors family genes including *Myf5* and *MyoD* [25]. The *MyoG* is a critical transcription factor in myogenesis and is essential for the terminal differentiation of committed myoblasts [26]. *FABP4* involves intracellular transport and fatty-acid metabolism [27]. The *LPL* has dual functions as a triglyceride hydrolase and a lipoprotein uptake [28]. Expression of *LPL* is recognized as a potentially important effector in lipoprotein metabolism, energy homeostasis, and body weight regulation [29]. Given the above review, dietary supplementation of TRP may be improved muscle differentiation, intracellular transportation of fatty acids, and catabolism of triglycerides in muscle by increasing the expression of *MYF6*, *MyoG*, *LPL*, and *FABP4*. The NEFA and TG showed no difference in serum between the control and treatment group ($p > 0.05$). However, the expression of *LPL* and *FABP4* gene were increased. The reason could be assumed as the muscle tissues utilize free fatty acids to myocyte differentiation and thus resulted in higher expression of the aforementioned gene.

TRP is reported to be the limiting AA in growing lambs and cattle during non-protein N use. Thus, supplementation of TRP in a rumen-protected form had positive effects on growth [6,8]. We verified that 74 feed ingredients contained TRP (average CP = 23% of DM, minimum CP = 3%, maximum CP = 95.5% of DM basis), and that 52 feed ingredients contain the lowest TRP in the midst of total AA composition include average TRP approximately 0.98% of total CP (minimum TRP = 0.44%, maximum TRP = 1.55 % of total CP) [5]. Dietary supplementation of 43.4 mg TRP per 1 kg of BW improves to increase the expression of gene related to muscle development, but its potent influence on muscle mass production remained unclear. It is mainly because we did not

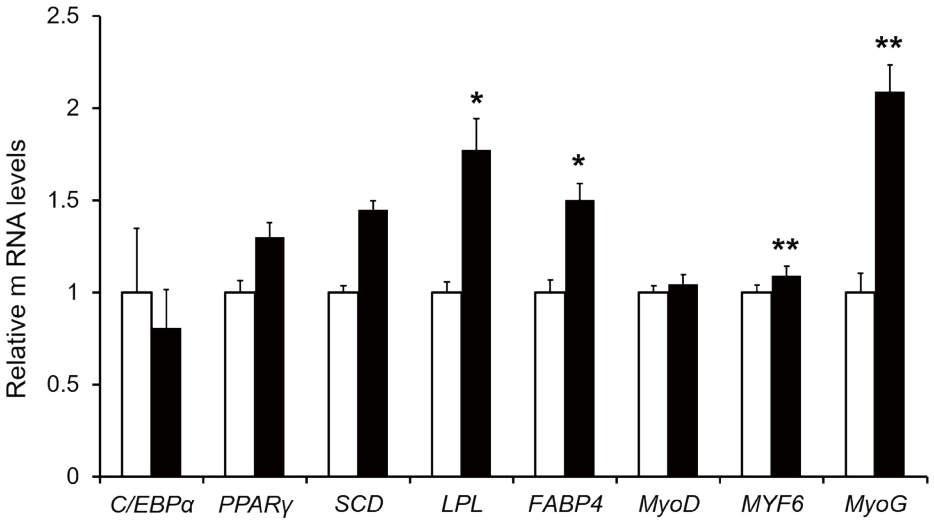

Fig. 1. Gene expression study in Hanwoo steers from control group and rumen-protected TRP-treated group. Validation by qPCR of 8 gene in *Longissimus dorsi* muscle. The qPCR values are shown as expression fold changes after normalization against the control *18S* rRNA, GAPDH, and RPLP0. □ Control group (n = 4), ■ Treatment group (n = 4). Data are presented as the means ± standard error. $^{*}p < 0.05$, $^{**}p < 0.01$. TRP, L-tryptophan; qPCR, quantitative polymerase chain reaction; C/EBPα, CCAAT/enhancer-binding protein alpha; PPARγ, peroxisome proliferator-activated receptor gamma; SCD, stearoyl-CoA desaturase; LPL, lipoprotein lipase; FABP4, fatty acid binding protein 4; MyoD, myoblast determination; MYF6, myogenic factor 6; MyoG, myogenine.

observe any changes in BW gain ($p > 0.05$, Table 3) and also, the carcass traits were not examined in this study. This study showed the possibility of muscle growth through gene expression including *MYF6*, *MyoG*, *FABP4*, and *LPL*. Although, there was no difference in growth performance by supplied 43.4 mg TRP per 1 kg of BW ($p > 0.05$), an increase in genes related to muscle development can indirectly improve muscle growth. In the future experiment, with larger sample size, long-term effects of TRP supplementation, and experiments with higher levels of TRP may influence growth performance or improve carcass traits to which are related to increasing muscle mass.

CONCLUSION

Dietary supplementation of TRP (43.4 mg kg^{-1} BW and 65 days in this study) may promote muscle development as observed in this study by increasing the expression of *MYF6*, *MyoG*, *FABP4*, and *LPL* gene. Further study with a larger sample size and higher amount of TRP supplementation is required to investigate possible changes in growth performance including carcass traits and blood metabolites and also to warrant the obtained results.

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