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Effects of dietary vitamin B_6 on the skeletal muscle protein metabolism of growing rabbits

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Abstract. This study aimed to evaluate the effects of dietary vitamin B_6 on the skeletal muscle protein metabolism and expression of transcription and growth factor of growing rabbits. Two hundred, healthy, rabbits with similar bodyweights were randomly assigned to one of five dietary groups with 40 animals per group. The dietary groups consisted of the following different vitamin B_6 supplementation levels: 0, 5, 10, 20 and 40 mg/kg. The feeding trial lasted 60 days. The results showed that dietary vitamin B_6 elicited significant effects on the fore and hind leg muscle ratio (the fore and hind leg muscle weight/the liveweight; $P < 0.05$) and on serum total amino acids (T-AA), blood urea and insulin-like growth factor 1 (IGF1) content ($P < 0.05$). Additionally, expression of *IGF1*, myogenic determination factor (*MYOD*) and myogenin (*MYOG*), myocyte regulation factor 5 (*MYF5*), myostatin (*MSTN*) and WW domain-containing E3 proteasome ubiquitin ligase 1 (*WWP1*) mRNA in the loin (*M. longissimus dorsi*) were affected by vitamin B_6 in diets ($P < 0.05$). The immunoblot analysis revealed that dietary vitamin B_6 elicited significant effects on IGF1, MYOG and WWP1 expression in the loin ($P < 0.05$). Our results indicate that the addition of dietary vitamin B_6 can significantly alter the protein metabolism of growing rabbits and that an appropriate vitamin B_6 supplementation level is 20 mg/kg for 3–5-month-old growing rabbits (the basic diet vitamin B_6 content was 4.51 mg/kg).

Additional keywords: serum constituents, slaughter performance.

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Introduction

Rabbit meat offers excellent nutritive and dietetic properties and is a common food in many Mediterranean countries and some other European countries (Dalle Zotte and Szendrõ 2011). Rabbit meat is rich in proteins, B vitamin and minerals, but it is low in sodium, fat and cholesterol. Thus, its energy content (789 kJ/100 g meat, average carcass value) is predominantly attributable to proteins (Dalle Zotte and Szendrõ 2011). From a nutritional perspective, rabbit meat is an ideal food for consumers, and is particularly useful in Western countries, where diets are generally rich in fats and sodium that promote health problems, such as obesity, cardiovascular diseases and hypertension (Karppanen and Mervaala 2006).

Vitamin B_6 is a member of the vitamin B complex group, which comprises a group of chemically similar compounds that can be interconverted in biological systems, and its active form, pyridoxal 5′-phosphate (PLP) is a co-factor for >120 enzyme-catalysed reactions. These include reactions in pathways for the synthesis and catabolism of amino acids and amines that act as neurotransmitters in the central nervous system (Clayton 2006). Lack of vitamin B_6 can cause animal anorexia, growth retardation, neurological disorders, anaemia and other symptoms and especially can lead to disorder of protein metabolism. Mauricio *et al*. (2000) used a method of isotope-labelling to investigate the effects of vitamin B_6 on protein turnover and reported that inadequate intake of vitamin B_6 elicits significant effects on one-carbon metabolism and overall protein turnover kinetics. Studies have also shown that when vitamin B_6 is supplemented in prawn feed, the dietary protein of diet can effect deposition *in vivo*, and the addition of higher concentrations of pyridoxine in Atlantic salmon feed can induce increased protein synthesis (Giri *et al.* 1997; Albektsen *et al.* 1998).

Nutritional factors are a major determinant of animal growth, and nutrients have been postulated to affect the expression of growth-regulatory genes, particularly those of the growth hormone/insulin-like growth factor (GH/IGF) axis have been postulated (Brameld 1997). IGF1 elicits extensive anabolic effects in various tissues, and malnutrition induced by food deprivation or restriction suppresses hepatic gene expression of IGF1 (Straus and Takemoto 1990; Pell *et al.* 1993; Weller *et al.* 1994; Sohlstrom *et al.* 1998) and, consequently, reduces circulating IGF1. In mammals, IGF1 signalling is predominantly mediated by the activation of the phosphoinositide 3-kinase (PI_3K)/serine/threonine kinase (AKT)/mammalian target of rapamycin (mTOR) pathway. The activation of the PI_3K/AKT/mTOR pathway promotes protein synthesis and muscle development (Glass 2003). Myogenic determining factor (MYOD), myogenin (MYOG) and myogenic factor 5 (MYF5) belong to a family of proteins known as myogenic regulatory

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factors (MRF). These basic helix–loop–helix transcription factors act sequentially in myogenic differentiation. MYOD is a tissue-specific MRF that acts as a master transcriptional switch for muscle differentiation and development, whereas MYF5 is the first myogenic regulatory protein expressed in the skeletal muscle lineage. In mammals, either MYOD or MYF5 is required for the formation of skeletal muscle (Rudnicki *et al.* 1993). Myostatin (MSTN), first discovered as a member of the growth differentiation factor family (GDF8), is a potent inhibitor of muscle growth and is expressed in embryonic and adult skeletal muscle (McPherron *et al.* 1997). Genetic deletion of MSTN leads to massive hyperplasia and hypertrophy of skeletal muscle in cattle (Kambadur *et al.* 1997; McPherron and Lee 1997), rats (Nishi *et al.* 2002), and humans (Schuelke *et al.* 2004). In chickens, both the proliferation and differentiation of embryonic myoblasts and the proliferation of satellite cells separated from skeletal muscle are inhibited by MSTN (Yang *et al.* 2003; McFarland *et al.* 2007). Whether MSTN mediates suppression of muscle development in growing rabbits remains unclear. Enhanced proteolysis in atrophying muscles is predominantly attributed to a general activation of the ubiquitin–proteasome (UBP) pathway. In mammals, the following three enzyme classes are involved in the UBP pathway: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3). The E3 are responsible for the recognition of substrates and their conjugation with ubiquitin (Pickart and Eddins 2004). The E3 play an important role in determining the proteins targeted for degradation by the proteasome. In mammals, IGF1 stimulates muscle growth by suppressing protein breakdown and the expression of muscle atrophy-related E3 (Sacheck *et al.* 2004). WW domain-containing E3 proteasome ubiquitin ligase 1 (WWP1), a HECT-type E3, has been suggested to be responsible for chicken muscular dystrophy (Matsumoto *et al.* 2008). However, whether WWP1 is involved in skeletal muscle protein metabolism of growing rabbits is unknown.

The effects of dietary vitamin B_6 on the skeletal muscle protein metabolism of growing rabbits are unknown. The objective of this study was to investigate and discuss the effects of vitamin B_6 on slaughter performance, serum biochemistry, serum hormone and skeletal muscle protein metabolism of growing rabbits. In addition, an appropriate vitamin B_6 supplemental level was also determined for 3–5-month-old growing rabbits.

Materials and methods

Chemical analyses of experimental diets

The experimental diet (Table 1) used in this study was formulated to meet the recommended nutrient requirements of growing rabbits (NRC 1977). The following five different concentrations were vitamin B_6 supplemented into the diets of the study animals: 0, 5, 10, 20, and 40 mg/kg (as-fed basis). The form of vitamin B_6 was pyridoxine hydrochloride (98%, Jiangxi Tyson Pharmaceutical Co., Ltd, Jiangxi, China). The five diets were passed through a roller mill before being mixed and granulated, pelletised (4–6 mm in length), and stored in the dark. All dietary analyses were performed in duplicate. Dry matter was determined by drying samples at 105°C to a constant weight. Vitamin B_6 in diets was measured using the VitaFast Vitamin B_6 assay kit

Table 1. Composition and nutrient levels of the experimental diet (air-dry basis, %)

Ingredients	Percentage	Chemical composition[B]	Content
Corn	15.0	Digestible energy (MJ/kg)	10.28
Soybean meal	10.0	Crude protein	16.20
Wheat bran	12.0	Crude fibre	17.47
Barley grain	10.0	Crude ash	11.75
Peanut straw	30.0	Ether extract	2.79
Sunflower meal	8.0	Lysine	0.60
Rice bran	10.0	Methionine	0.27
Premix[A]	5.0	Calcium	0.97
Total	100.0	Phosphorus	0.43

[A]Premix provided the following nutrients per kg of diets, vitamin A: 10 000 IU; vitamin D_3: 2000 IU; vitamin E: 50 mg; vitamin K_3: 2.5 mg; vitamin B_1: 5 mg; vitamin B_2: 10 mg; nicotinic acid: 20 mg; pantothenic acid: 50 mg; folic acid: 2.5 mg; vitamin B_{12}: 1 mg; choline chloride: 400 mg; Fe: 100 mg; Zn: 50 mg; Cu: 40 mg; Mn: 30 mg; I: 0.5 mg; Se: 0.05 mg; $CaHPO_4$: 15 000 mg; NaCl: 5000 mg; lysine: 1, 500 mg; methionine: 1, 500 mg; 10% bacitracin zinc: 300 mg; the rest is miscellaneous meal carrier complement.

[B]Digestible energy was calculated according to 'Tables of feed composition and nutritive values in China' (Chinese Academy of Agricultural Sciences 2009), whereas the others were measured values.

(R-Biopharm AG, Darmstadt, Germany), and levels were 4.51, 9.66, 14.64, 24.32, and 44.81 mg/kg (air-dry basis). The Association of Official Analytical Chemists (AOAC) International procedures (AOAC International 2005) were used to determine the content of ash (942.05). Crude fibre was determined according to procedure 932.09 of AOAC (AOAC International 1995), and crude protein and ether extract were determined according to procedure 968.06 and 920.39 of AOAC (AOAC International 2000), respectively. Crude protein content (6.25 × N) and ether extract were determined using a Kjeltec Auto 1030 Analyser and Soxtec 1043, respectively (FOSS Tecator AB, Höganäs, Sweden). Lysine and methionine of the feed were analysed using an automatic amino-acid analyser (High-speed Amino Acid Analyzer, Basic L-8900, Tokyo, Japan).

Animals and experimental design

In this study, 200, healthy, 3-month-old growing Rex rabbits of similar bodyweight (1682 ± 40 g) were randomly assigned to one of the five diets, with 40 animals per dietary group. The experimental procedures were approved by the Committee of Ethics in Research of Shandong Agricultural University and performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China). The 60-day feeding trial included a 7-day adjustment period and a 53-day experimental period. The experimental rabbits were housed singly in a cage (60 × 40 × 40 cm) and had *ad libitum* access to food and water. The animals were housed in a semi-controlled closed building during the experimental period at 18–25°C.

Sample collection and preparation

At the end of the trial, 40 rabbits (8 rabbits per treatment, 4 male and 4 female, with their bodyweights around the average group bodyweight) were bled by cardiac puncture after the animals

fasted for 12 h at ~1500 hours for blood samples and then were sacrificed by exsanguination from the carotid artery. The blood samples were centrifuged at a centrifugal force 1500 for 10 min. The isolated serum samples were stored at −20°C for further analysis. Simultaneously, the loin (*M. longissimus dorsi*) muscle samples were also collected, frozen immediately in liquid nitrogen and subsequently stored at −80°C, for subsequent analysis.

Determination of indicators and methods

Determination of slaughter performance

Twelve hours before slaughter, the rabbits were fasted and weighed (for ‘liveweight’). The slaughter procedure and carcass analysis were performed as described by Blasco and Ouhayoun (1996). After bleeding, the pelts, paws and full gastrointestinal tract were removed, and the hot carcasses were weighed. The slaughter ratio was calculated by dividing the hot dressed carcass weight (including the head, thoracic cage organs, liver, kidneys and perirenal fat) with the liveweight before slaughter and expressed as a percentage. The eviscerated ratio was the percentage of eviscerated carcass weight (hot carcass weight without head and any internal organs)/the liveweight before slaughter. The loin muscle, fore leg muscle and hind leg muscle masses were weighed and their proportion to the liveweight was calculated.

Determination of serum biochemistry

A sequential multiple analyser (Hitachi 7020, Tokyo, Japan) was used to analyse serum glucose, urea, alkaline phosphatase (AKP), calcium ion (Ca^{2+}) and inorganic phosphorus (IP) following the manual of commercial protocols (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). Serum total amino acids (T-AA) using a T-AA assay kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China) determined.

Determination of serum hormone

Serum growth hormone (GH), insulin (INS) and insulin-like growth factor 1 (IGF1) were analysed using commercial Radioactive Immune Assay Kits supplied by Tianjin Jiuding Co. (Tianjin, China), and radioactivity was determined in DFM-96 10 tubes in a Radioactive Immune gamma counter.

Determination of related gene mRNA expression

Total RNA was extracted from the loin muscle samples by a single-step isolation procedure using Trizol reagent (Invitrogen Corporation, New York, NY, USA). The RNA concentrations were determined by measuring absorbances at 260 nm. Two micrograms of total RNA was used to synthesise cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany), cDNA samples were stored at −20°C.

Semi-quantitative real-time–polymerase chain reaction (PCR) was performed to determine the expression levels of *IGF1*, *IGF1* receptor (*IGF1R*), *MYOD*, *MYOG*, *MYF5*, *MSTN* and *WWP1*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. All quantitative PCR primers (Table 2) were designed by Primer Premier 5 software and synthesised by SANGON Biological Engineering Co., Ltd (Shanghai, China). PCR amplification was performed using Fast

Table 2. Primer sequence

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *IGF1*, insulin-like growth factor 1; *IGF1R*, insulin-like growth factor 1 receptor; *MYOD*, myogenic determining factor; *MYOG*, myogenin; *MYF5*, myocyte regulation factor 5; *MSTN*, myostatin; *WWP1*, WW domain-containing E3 proteasome ubiquitin ligase 1. The primers were designed by Primer Premier 5 software, synthesis by SANGON Biological Engineering Co., Ltd (Shanghai, China)

Gene	GenBank accession number	Specific primers	Product size (bp)
GAPDH	NM_001082253	F:5′-TGCCACCCACTCCTCTACCTTCG-3′ R:5′-CGAAGGTAGGGATGGGTGGCA-3′	118
IGF1	NM_001082026	F:5′-TCTGAGGAGGCTGGAGATGT-3′ R:5′-TGTTGGTAGATGGAGGCTGA-3′	122
IGF1R	XM_008248789	F: 5′-AAGGGCGACATAAACACCAG-3′ R:5′-AGGTTAGGATGATGCGGTTC-3′	106
MYOD	NM_001171407	F: 5′- GCTTCAACCTTCCGCACAG-3′ R:5′-CACCTCCATTGCTCAGACCT-3′	86
MYOG	NM_204184	F: 5′-TCAAGGTAACGGACACACCA-3′ R:5′-CGCAATAATCCAATCCCATC-3′	140
MYF5	XM_002711365	F:5′-TTAACCAGGCTTTCGAGACG-3′ R:5′-TCTCCACCTGCTCTCTCAGC-3′	135
MSTN	NM_001109821	F: 5′-TCAAGGTAACGGACACACCA-3′ R:5′-CGCAATAATCCAATCCCATC-3′	137
WWP1	XM_002710674	F: 5′-TGAACAGTGGCAATCTCAGC-3′ R:5′-CTGGTGGCAAAGGTCCATA-3′	122

Start Universal SYBR Green Master (Roche Diagnostics GmbH). The volume of each PCR reaction was 20 µL, including 2 µL cDNA, 10 µL SYBR Premix Ex *Taq* (×2), 0.5 µL PCR Forward Primer (10 µM), 0.5 µL PCR Reverse Primer (10 µM), 0.4 µL ROX Reference Dye II (×50) and 6.6 µL ddH_2O. The number of cycles set for the linear amplification of the cDNA was 40. All samples were run in duplicate, and the standard curves were generated using pooled cDNA from the samples being assayed. The amplified products were analysed by electrophoresis in 1.0% agarose gels. The data were normalised to the internal control *GAPDH*, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

western blot analyses

Protein was extracted from the loin muscle samples using mortar and pestle, and protein concentrations were determined using a BCA Protein Assay Kit (Beijing Kangwei Century Biotechnology Co., Ltd, China). We selected four proteins IGF1, MYOG, MSTN and WWP1, both of which were subject to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The extracted proteins (50 ng/sample) were solubilised in 40 mmol SDS-loading buffer (Beijing Solarbio Science and Technology Co., Ltd, China) and then resolved by electrophoresis (Bio-Rad, Richmond, CA, USA) and 12.5% SDS-PAGE before being transferred electrophoretically to a polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The standard markers for protein molecular masses were purchased from Thermo Corporation (New York, NY, USA). The membranes were blocked with 5% skimmed milk in phosphate buffer solution (Beijing Solarbio Science and Technology Co., Ltd) at 4°C overnight and incubated with primary antibodies (IGF1 antibody orb125044, Biorbyt Ltd, Cambridge, UK). Anti-MYOG/Myogenin antibody (C-Terminus) LS-C113013, Anti-MSTN/GDF8/Myostatin antibody (aa301–350) LS-C186740 and Anti-WWP1 antibody (N-Terminus) LS-C110755 1 : 200 dilution, Life Span BioSciences, Inc., New York, NY, USA). The membranes were then rinsed in Tris Buffered Saline Tween (Beijing Solarbio Science and Technology Co., Ltd), and subjected to detection with 1 : 3000 diluted horse radish peroxidase-conjugated goat anti-mouse IgG antibody (Beijing Dingguo Changsheng Biotech Co. Ltd, China) at 37°C for 1 h. Proteins were visualised using BeyoECL reagents (Beyotime, Shanghai, China). The intensity of the bands was quantified by a Pro Plus 6.0 Biological Image Analysis System IGF1, MYOG, MSTN and WWP1 were normalised to the internal control β-tubulin, and the relative expression levels were calculated.

Statistical analyses

The data were analysed using the GLM procedure of SAS 9.1 statistical software, and were expressed as the mean values and root mean square error, $P < 0.05$ was considered to be significant.

Results

Effect of vitamin B_6 on slaughter performance

The effects of dietary vitamin B_6 on slaughter traits are shown in Table 3. Dietary vitamin B_6 affected the fore leg muscle ($P = 0.0010$) and hind leg muscle ($P = 0.0295$) ratios. Vitamin B_6 did not affect the slaughter, eviscerated or loin muscle ratios ($P > 0.05$).

Effect of vitamin B_6 on serum biochemistry

The serum biochemistry analysis results are shown in Table 4. Dietary vitamin B_6 had significant influences on the content of serum T-AA and urea ($P = 0.0481$ and $P = 0.0358$, respectively), and highest T-AA or lowest urea measurements were observed in the 20 mg/kg group. Vitamin B_6 did not affect glucose, AKP, Ca^{2+} or IP concentrations ($P > 0.05$).

Effect of vitamin B_6 on serum hormones

There were no overall differences in serum GH and INS ($P = 0.4311$ and $P = 0.0685$, respectively). Vitamin B_6 elicited significant influence on serum IGF1 concentration ($P = 0.0423$, Table 5), which increased with vitamin B_6 levels.

Effect of vitamin B_6 on related gene mRNA expression

As shown in Fig. 1, dietary vitamin B_6 elicited significant influence on *IGF1*, *MYOD*, *MYOG*, *MYF5*, *MSTN* and *WWP1* ($P < 0.05$), and no significant effects were observed on *IGF1R* mRNA expression ($P > 0.05$).

Table 3. Effects of dietary vitamin B_6 on slaughter performance of growing rabbits

a, b, Different letters denote significance ($P < 0.05$). $n = 8$ per group. R-MSE, root mean square error

Items	Dietary vitamin B_6 supplemental level (as-fed basis; mg/kg)					R-MSE	*P*-value
	0	5	10	20	40		
Slaughter ratio[A] (%)	85.9	85.7	86.0	85.6	86.2	1.03	0.7875
Eviscerated ratio[B] (%)	54.5	54.1	56.1	57.0	56.2	1.94	0.0913
Loin muscle ratio[C] (g/kg)	111.8	121.0	130.2	126.9	116.1	14.01	0.0771
Fore leg muscle ratio[C] (g/kg)	101.7c	114.5b	122.1a	121.6a	115.5b	9.60	0.0010
Hind leg muscle ratio[C] (g/kg)	207.3b	220.7ab	238.3a	233.6a	238.4a	21.83	0.0295

[A]Hot carcass weight (including the head, thoracic cage organs, liver, kidneys and perirenal fat) as a percentage of liveweight.

[B]Eviscerated carcass weight (hot carcass weight without head and any internal organs) as a percentage of liveweight.

[C]The loin (*M. longissimus dorsi*) muscle, fore leg muscle and hind leg muscle masses were weighed and their proportion to the liveweight was calculated.

Table 4. Effects of dietary vitamin B_6 on serum biochemistry of growing rabbits

a,b, Different letters denote significance ($P < 0.05$). $n = 8$ per group. T-AA, total amino acids; AKP, alkaline phosphatase; Ca^{2+}, Calcium ion; IP, inorganic phosphorus; R-MSE, root mean square error

Items	Dietary vitamin B_6 supplemental level (as-fed basis; mg/kg)					R-MSE	*P*-value
	0	5	10	20	40		
Glucose /(mmol/L)	6.89	5.59	7.36	7.16	7.84	1.643	0.6232
T-AA/(mmol/L)	11.73b	12.26ab	13.99ab	17.17a	14.49a	8.395	0.0481
Urea/(mmol/L)	4.48a	4.33ab	3.93b	3.79b	3.86b	0.635	0.0358
AKP/(U/L)	136.06	148.00	139.13	119.31	109.75	46.608	0.4798
Ca^{2+}/(mmol/L)	2.95	2.92	3.06	2.93	3.05	0.220	0.5212
IP/(mmol/L)	2.20	2.13	2.24	1.95	2.30	0.463	0.6026

Table 5. Effects of dietary vitamin B_6 on serum hormones of growing rabbits

a,b, Different letters denote significance ($P < 0.05$). $n = 8$ per group. GH, growth hormone; INS, insulin; IGF1, insulin-like growth factor 1; R-MSE, root mean square error

Items	Dietary vitamin B_6 supplemental level (as-fed basis; mg/kg)					R-MSE	*P*-value
	0	5	10	20	40		
GH/(ng/mL)	49.12	49.85	50.34	51.76	51.32	6.955	0.4311
INS/(uIU/mL)	4.68	5.10	5.94	7.18	9.10	4.265	0.0685
IGF1/(ng/mL)	214.56c	274.75b	276.53b	274.93b	292.12a	23.925	0.0423

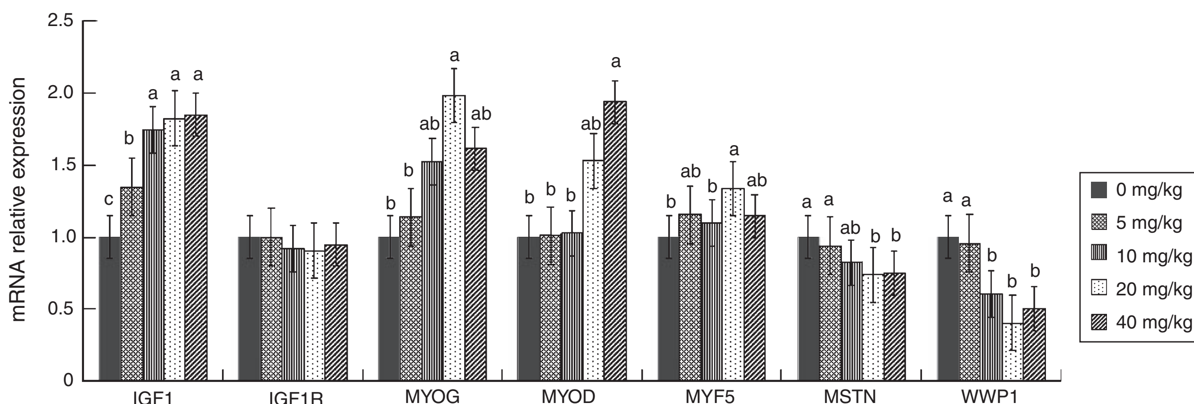

Fig. 1. Dietary vitamin B_6 on the relative expression of mRNA in skeletal muscle of growing rabbits. IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; MYOD, myogenic determining factor; MYOG, myogenin; MYF5, myocyte regulation factor 5; MSTN, myostatin; WWP1, WW domain-containing E3 proteasome ubiquitin ligase 1. Total RNA was extracted from the loin (*M. longissimus dorsi*) muscle samples by a single-step isolation procedure using Trizol reagent (Invitrogen Corporation, New York, NY, USA). The values are expressed as the means ± standard error, and $n = 8$ per group. Dietary vitamin B_6 supplemental levels of 0, 5, 10, 20, and 40 mg/kg were incorporated in the feed. The means in the same graph with unlike letters differ ($P < 0.05$).

Effect of vitamin B_6 on related protein expression

To verify the accuracy of the fluorescence quantitative PCR results to further validate the difference in the protein profile of the loin muscle tissue from differently treated rabbits, IGF1, MYOG, MSTN and WWP1 were selected for western blotting, SDS-PAGE and western blot of experimental rabbits skeletal muscle proteins are shown in Figs 2 and 3, the relative expression of IGF1, MYOG were increased, whereas that of WWP1 was decreased by dietary treatment of vitamin B_6 ($P < 0.05$). In contrast, the abundance of MSTN was unaffected by vitamin B_6 (Fig. 4).

Discussion

Effect of vitamin B_6 on slaughter performance

The effect of diet composition on slaughter traits has been thoroughly investigated. Slaughter and eviscerated ratios are the main indicators to measure meat production performance of livestock. In general, slaughter and eviscerated ratios above 80% and 60%, respectively, are indicators of optimal meat performance of land animals. Animal growth is predominantly due to the increases in protein synthesis. The leg muscles are the most highly organised organs of protein deposition in mammals

and are also the most representative of animal growth. Previous studies show that vitamin B_6 is essential for the growing Rex rabbit, and it can significantly influence on average daily gain and average daily feed intake. To maintain maximum growth its requirement for this vitamin was established to be 20 mg/kg (Liu *et al.* 2015). In this study, dietary vitamin B_6 levels elicited no significant influences on slaughter or eviscerated ratios. This finding is in agreement with other studies that found no significant differences of the carcass net weight or eviscerated ratio from rabbits that were slaughtered at 2500 g in weight (Cobos *et al.* 1995), the diets did not significantly influences the carcass yield of the rabbits (Peiretti and Meineri 2008). However, the role of vitamin B_6 in amino acid and nucleic acid metabolism suggests that inadequate dietary vitamin B_6 might impair protein synthesis. Sampson *et al.* (1988) demonstrate that marginal vitamin B_6 intake alters protein synthesis in the liver, kidney and skeletal muscle of the rat. This study suggests that dietary vitamin B_6 levels can enhance muscle yield, which might be attributed to PLP promote the metabolism of protein synthesis.

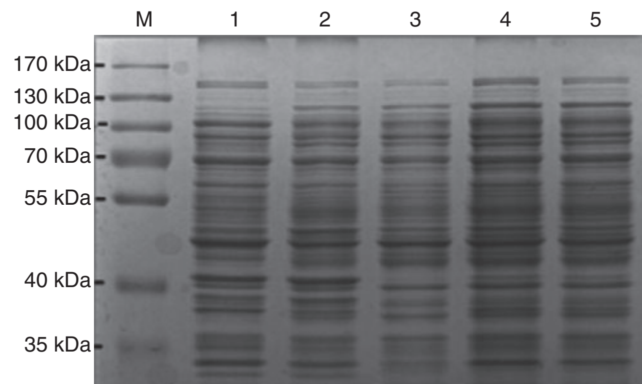

Fig. 2. SDS-PAGE map of experimental rabbit skeletal muscle protein. M: Pre-dyed protein marker; 1, 2, 3, 4, 5 correspond to 0, 5, 10, 20, 40 mg/kg group. Protein extracted from the loin (*M. longissimus dorsi*) muscle samples.

Effect of vitamin B_6 on serum biochemistry

Malmolf (1998) reported that the urea concentrations precisely reflect the balance between protein and amino acid metabolism. The level of dietary amino acids was optimal when the urea concentration was the lowest (Coma *et al.* 1995, 1996). In this study, urea concentrations were decreased significantly when the dietary vitamin B_6 levels were increased. According to the study, the minimal urea concentration was obtained at 20 mg/kg. Therefore, using urea as a criterion, the optimal vitamin B_6

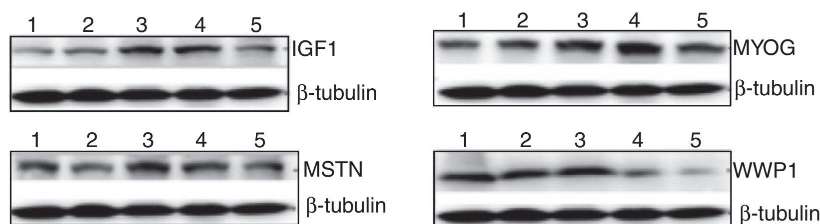

Fig. 3. Western blot of experimental rabbit target proteins in skeletal muscle. IGF1, insulin-like growth factor 1; MYOG, myogenin; MSTN, myostatin; WWP1, WW domain-containing E3 proteasome ubiquitin ligase 1. 1, 2, 3, 4, 5 correspond to 0, 5, 10, 20, 40 mg/kg group. Protein extracted from the loin (*M. longissimus dorsi*) muscle samples. The expression of IGF1, MYOG was increased, whereas that of WWP1 was decreased by dietary vitamin B_6 supplementation levels.

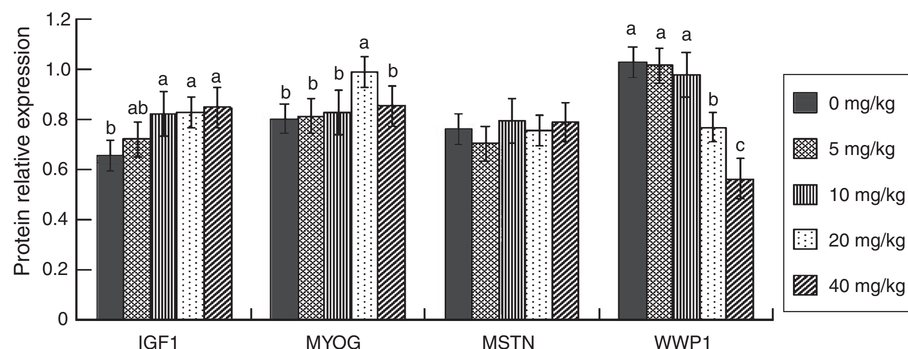

Fig. 4. Dietary vitamin B_6 on the relative expression of proteins in skeletal muscle of growing rabbits. IGF1, insulin-like growth factor 1; MYOG, myogenin; MSTN, myostatin; WWP1, WW domain-containing E3 proteasome ubiquitin ligase 1. Protein extracted from the loin (*M. longissimus dorsi*) muscle samples. Values expressed as the mean values ± standard error, and $n = 8$ per group. Dietary vitamin B_6 supplemental level 0, 5, 10, 20, and 40 mg/kg were incorporated into the feed. The mean values in the same graph with unlike letters differ ($P < 0.05$).

level for growing rabbits from 3 to 5 months old was 20 mg/kg. The main result of supplementing growing rabbits with vitamin B_6 in our study was to increase serum T-AA concentrations and to decrease serum urea concentrations. Due to the integral roles of PLP in skeletal muscle protein synthesis, the underlying mechanism needs to be investigated further. In this study, dietary vitamin B_6 levels appeared to elicit no effect on serum glucose concentrations in growing rabbits, the serum INS concentration was also unaffected by dietary vitamin B_6 levels, the lack of effects on glucose might be attributed to the energy and protein in the diets that met the dietary requirements of the rabbits, and INS can regulate blood glucose concentrations. Additionally, serum AKP, Ca^{2+} and IP concentrations were not affected by dietary vitamin B_6 levels. These results suggest that PLP is not involved in the metabolism of calcium and phosphorus.

Effect of vitamin B_6 on serum hormones

Pituitary GH is the principal hormone that regulates postnatal growth in animals. Studies have reported that in most species, nutritional restriction leading to growth suppression is accompanied by an increase in serum GH concentrations (Soliman *et al.* 1986; Buonomo and Baile 1991; Vance *et al.* 1992). Plasma GH concentrations can be elevated by malnutrition (Breier 1999), which is related to attenuated negative feedback within the somatotropic axis and decreased hypothalamic somatostatin secretion. In this study, a significant difference in serum GH concentrations among treatments was not observed as dietary vitamin B_6 levels increased, which was in agreement with the previous studies reporting that increased dietary vitamin B_6 levels or vitamin B_6 deficiency does not affect plasma GH concentrations (Buonomo and Baile 1990). INS and IGF1 elicit extensive anabolic effects in various tissues. Takahashi *et al*. (1990) demonstrated that rats fed protein-free diets exhibited a lower plasma IGF1 concentrations. Plasma concentrations of IGF1 increase significantly with increasing nutrient intake (Smith *et al.* 2002). Previous studies concluded that the circulating IGF1 concentrations are sensitive to nutritional changes. In our experiments, the serum INS concentrations were also unaffected by dietary vitamin B_6 levels, although there was a trend of increasing serum INS concentrations from 0 to 40 mg/kg. The lack of effects of dietary vitamin B_6 on INS might be because that the diets were formulated to contain constant levels of digestible energy and crude protein so that the energy and protein requirements of the growing rabbits were met, it was also confirmed by serum glucose concentrations. There was a significant influence on serum IGF1 concentrations by increasing with dietary vitamin B_6 levels. Stick *et al*. (1998) showed that serum IGF1 concentrations in cattle are positively correlated with average daily gain, and in this study, the IGF1 concentrations were also positively correlated with slaughter performance. These results support the hypothesis that IGF1 plays a role in the growth and in efficiency of feed utilisation in growing rabbits.

Effect of vitamin B_6 on skeletal muscle protein metabolism

Nutrient availability is among the most important environmental variable affecting muscle growth (Valente *et al.* 2012). IGF1 is a key regulator of muscle development and metabolism in vertebrate species, can promoting muscle growth (Glass 2005). IGF1 increases muscle *MYOD* and *MYOG* gene expression, as well as proliferation and differentiation of satellite cells (Florini *et al.* 1996). An increase in *IGF1R* mRNA in skeletal muscle has been reported in cardiac cachexia (Schulze *et al.* 2003), as well as in cancer cachexia (Costelli *et al.* 2006). Besides, in these experimental models of muscle wasting, a decrease in *IGF1* gene expression in the skeletal muscle was observed. In this study, an increasing in *IGF1*, *MYOD* and *MYOG* mRNA expression was observed, but no significant effect on *IGF1R* in the skeletal muscle, the mechanism might be PLP decrease the muscle wasting and increase muscle growth.

MSTN is a growth factor of the transforming growth factor family that negatively regulates skeletal muscle growth. An increase in *MSTN* gene expression has been reported in muscular wasting associated with several conditions such as glucocorticoid administration (Ma *et al.* 2003; Gilson *et al.* 2007). Furthermore, inactivation of the *MSTN* gene results in an increase in skeletal muscle mass (Lee and McPherron 2001). The effects of MSTN on skeletal muscle include inhibition of protein synthesis and *MYOD* expression (McFarlane *et al.* 2006). The upregulated *IGF1* and downregulated *MSTN* mRNA levels in vitamin B_6-treated rabbits indicate that IGF1 and MSTN are involved in the regulation of muscle development. In line with these results, there is a positive relationship between muscle *IGF1* mRNA levels and the post-hatch muscle growth of chickens (Duclos 2005). However, both proliferation and differentiation were found to be inhibited by MSTN in chick embryonic myoblasts (Yang *et al.* 2003). The different observation regarding IGF1 and MSTN is in line with the result in mammals that MSTN expression is not influenced by IGF1 deletion (Miyake *et al.* 2007), suggesting that no strong correlation exists between IGF1 and MSTN. In this experiment, vitamin B_6 supplementation significantly reduced *MSTN* mRNA expression in rabbit muscles, but MSTN protein levels were found to be unchanged after treatment with vitamin B_6, the underlying mechanism needs to be investigated further.

In the present study, the effects of vitamin B_6 on the expression of myogenic factors were investigated. MYOD, MYF5 and several MRFs initiate and maintain the expression of muscle-specific genes during embryogenesis and postnatal muscle growth (Bailey *et al.* 2001). In rats, the loss of MRF results in reduced body size (Knapp *et al.* 2006). MYOD acts as an early myogenic transcription factor, mainly involved in satellite cell activation and proliferation, whereas MYOG is a late-acting myogenic regulatory factor, expressed during differentiation. A decrease in MYOD and MYOG has been observed in muscle wasting induced by cancer and experimental cirrhosis (Costelli *et al.* 2005; Dasarathy *et al.* 2007). In this study, upregulated transcription of *MYOD*, *MYOG* and *MYF5* in response to the dietary vitamin B_6 level was observed in growing rabbits, indicating that vitamin B_6 is involved in the process of muscle protein metabolism related to myogenic factors.

In mammals, there is accumulating evidence indicating the involvement of the UBP pathway in muscle wasting during pathophysiological conditions. In this study, the expression of WWP1 was also affected by dietary nutrient levels, in the experimental diets supplementation vitamin B6 significantly

reduced the expression of WWP1 in rabbit muscles, suggesting that UBP pathway may be involved in skeletal muscle protein metabolism by vitamin B_6 treatment. According to previous studies in rabbits, one of vitamin B6 inhibit proteolysis is might by UBP pathway. In summary, dietary vitamin B_6 can improve the utilisation ratio of protein, promotes skeletal muscle protein synthesis and inhibits decomposition was also confirmed by reduced serum urea concentrations.

Conclusion

Dietary vitamin B_6 elicited effects on rabbit muscle deposition in growing rabbits, and one of the potential mechanisms for vitamin B_6 in improving slaughter performance in growing rabbits was via increasing the synthesis and secretion of IGF1. For feed predominantly composed of corn, wheat bran and peanut straw, the most appropriate dietary vitamin B_6 content for 3–5-month-old growing rabbits was 20 mg/kg (the basic dietary vitamin B_6 content was 4.51 mg/kg).

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