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Intensive Nursing and Feeding During the Early Growth Period Altered Intramuscular Adipogenesis in Crossbred Steers (Japanese Black Male x Holstein Female)

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Abstract: We investigated the influence of feed quality during the early growth stage on expression of adipogenic genes in skeletal muscle of crossbred cattle. The cattle were divided three groups at 45 days of age group high energy (H: n = 7), group normal energy (N: n = 7) and group roughage (R: n = 7). Cattle in group H were three fold-intensively fed a high protein and low fat milk replacer (Crude Protein (CP): 28%, Ether Extracts (EE): 18%, max: 2.0 kg, 12 L day^{-1}) from 45 days to 4 months of age. In groups N and R, cattle were fed a normal milk replacer (CP: 25%, EE: 23%, max: 0.5 kg, 4 L day^{-1}) from 45 days to 4 months of age. From 4-10 months of age, cattle in group H were fed a high-nutrition Total Mixture Rate (TMR), while group N were fed a normal-nutrition TMR and group R were fed only roughage. Muscle samples were taken by biopsy from *Longissimus thoracis* muscle (LT) at 45 days, 4 and 10 months of age. Results from assays using quantitative real-time Polymerase Chain Reaction (PCR) revealed that gene expression of Peroxisome Proliferator-activated Receptor Gamma 2 (PPARγ2), CCAAT/Enhancer-Binding Protein alpha (C/EBPα), Stearoyl-CoA Desaturase (SCD), Glucose-6-Phosphate Dehydrogenase (G6PD), Fatty Acid Synthase (FASN) and Leptin related to adipogenesis and fatty acid synthesis differed among groups. The expression of these genes was significantly different among groups at 10 months but not at 4 months of age. These results demonstrated that differences in feed quality during early growth stages had substantial effects on the expression of genes related to adipogenesis and fatty acid synthesis in LT.

Key words: Crossbred cattle, intensive nursing, feed quality, adipogenesis, muscle, gene expression

INTRODUCTION

Nutrient composition and the quantity of milk replacer have a significant impact on body composition in calves. In previous studies, Jasper and Weary (2002) reported that *ad libitum* feeding of milk to dairy calves led to increased weight gain and Blome *et al*. (2003) examined the relationship between diet composition and body growth in male Holstein calves. These reports demonstrated that increasing the crude protein in milk replacers linearly increased the growth rate of calves. Furthermore, Bascom *et al*. (2007) reported that the benefit of feeding programs in Jersey bull calves involving intensified protein was increased weight gain without increased fat deposition. The study also showed that feeding with a higher-protein milk replacer improved feed efficiency and increased the rate of weight gain during the pre-weaning period in Holstein heifer calves (Brown *et al*., 2005; Cowles *et al*., 2006). Bartlett *et al*. (2006) and Diaz *et al*. (2001) also reported an increase in body fat, Average Daily Gain (ADG) and feed efficiency when the rate of milk replacer feeding was increased. Moreover, Abdelsamei *et al*. (2005) suggested that a high level of milk intake during the pre-weaning phase resulted in heavier weaning weights and that protein and fat concentrations in the carcass and empty body weight at slaughter age (they reached an ultrasonic lipid concentration of 4-5% in the 12th rib) increased linearly with peak milk levels in spite of the same post-weaning feeding regime. Thus, feeding quality (high level of milk

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intake) during the pre-weaning period could markedly affect the body composition of cattle and could decrease the number of days to the slaughter weight at which a similar rib lipid concentration is reached.

Intramuscular fat content, or marbling of cattle muscle is an important component of traits that influence eating quality (Hovenier *et al*., 1993). Animals of the Japanese Black (or Wagyu) breed of cattle are genetically predisposed to accumulate intramuscular fat (Zembayashi *et al*., 1995; Gotoh *et al*., 2009). Therefore, in Japan some dairy farmers crossbreed Japanese Black males with Holstein females to obtain more valuable calves and these crossbred cattle (Japanese Black male x Holstein female: F1) are in fact useful for meat production. The meat quality of F1 is superior to that of Holstein and the meat quantity is greater than that of Japanese Black. Although, the meat quality of F1 is inferior to that of Japanese Black it is satisfactory for the Japanese beef market. Furthermore, daily gain is greater and the fattening efficiency is also improved. As a reasonable source of beef, F1 as a by-product from dairy farms is more acceptable than Japanese Black in the market. Therefore, it is important to establish a technique for improving meat quality and quantity in the beef production system for F1.

The potential for cellular development of adipocytes is believed to be fixed relatively early in life (Pethick *et al*., 2004) and so it is important to develop the intramuscular fat in early life. Abdelsamei *et al*. (2005) suggested that calves consuming increased amounts of milk replacer and nursed for a longer period may develop intramuscular adipocytes. These results show that nutritional states during the early growth period have a great impact on final fat cell development. However, the relationship between nutritional states and adipogenic gene expression in the early developmental stages of F1 is as yet unknown.

Therefore, the objective of this study was to investigate the influence of intensified calf nursing and feed quality during the early growth stages on mRNA expression of adipogenic genes in the LT of F1.

MATERIALS AND METHODS

Animals and groups: Twenty-one crossbred steers (Japanese Black male x Holstein female) were used in this experiment. Briefly, the crossbred steers with initial Body Weight (BW) of 50.0±5.9 kg were randomly allocated to three groups. The group H (n = 7) were intensively fed a high protein and low fat milk replacer (CP: 28%, EE: 18%, max: 2.0 kg, 12 L day^{-1}) daily from 45 days to 4 months of age. After weaning, they were fed a high nutrition Total Mixture Rate (TMR) until 10 months of age. The composition of the TMR was Total Digestible Nutrients (TDN): 73-76%, CP: 19-17% and Neutral Detergent Fiber (NDF): 29-30%. The group N (n = 7) and group R (n = 7) were fed a normal milk replacer (CP: 25%, EE: 23%, max: 0.5 kg, 4 L day^{-1}) daily from 45 days to 4 months of age. Subsequently, the N group were fed a normal nutrition TMR (TDN: 70-72%, CP: 18-16% and NDF: 37-38%). Group R was restrictively fed Alfalfa meal (TDN: 56%, CCP: 19%, CNDF: 44%) and *ad libitum* Timothy hay (TDN: 60%, CP: 10%, NDF: 64%) until 10 months of age. In groups H and N, the fed TMR was increased gradually to avoid dramatic changes in nutritional levels during the experimental period. On the other hand, in group R, the steers were initially fed restrictively starter (1.0 kg) to reduce stresses against weaning, for the first two weeks after weaning. All steers were raised using a tethering system from 5-10 months of age and were permitted free access to mineral salt blocks and water. Groups N and R were treated under the same conditions from biopsy time point 1 (BP1) to BP2 therefore, the two groups were combined until BP2 (N and R). There were no detectable adipocytes in samples taken at BP1.

Skeletal muscle samples were taken from LT by needle or shot biopsy, three times from every animal at commencement of the experiment (45 days of age, BP1), towards the end of the weaning point (4 months of age, BP2) and near the end of nutritional treatment (10 months of age, BP3, Table 1). At BP1, needle biopsy was used and 8 cm skeletal muscle samples were taken for

Table 1: Body weight changes at different biopsy time points (BP1-BP3)

	Biopsy time point		
Body weight (kg)	BP1 (45 days*)	BP2 (4 months*)	BP3 (10 months*)
Group H	53±7	179±21a	372±29A
Group N	53±5	149±17b	331±56B
Group R	-	-	279±13C

$^{A-C}$Values with different letters among groups are significantly different (p<0.01); ^{a,b}Values with different letters among groups are significantly different (p<0.05); *Months: Months of age the High energy group (H) was fed with intensive nursing and high energy concentrate feeding, the Normal energy group (N) was fed with normal nursing and normal energy concentrate feeding and the Roughage group (R) was fed with normal nursing and roughage feeding until 10 months of age; BP1, BP2 and BP3 indicate sampling time points of biopsy; Results are means±SD

Table 2: Primer information for quantitative RT-PCR

Gene of interest	Gene symbol	Accession No.	Sense[1]	Primer sequence (5'-3')	Size (bp)
Peroxisome proliferator-activated receptor gamma 2	PPARγ2	Y12420	FOR	ctgttccgtgctgtgatggg	192
			REV	ggcatgggagtggtcatccatc	
CCAAT/enhancer-binding protein alpha	C/EBPα	DQ068270	FOR	gctgaccagtgacaatgacc	109
			REV	cttgaccagggagctctcg	
Stearoyl-CoA desaturase	SCD	AB075020	FOR	cgacctaagagccgagaagc	195
			REV	gcagcactattcaccagccag	
Glucose-6-phosphate dehydrogenase	G6PD	NC_007331	FOR	gccagtacgatgacactgc	158
			REV	cgtctggctcatgcaggtc	
Fatty acid synthase	FASN	AY343889	FOR	ctaccaagccaggcaggtc	226
			REV	gccattgtacttgggcttgt	
Leptin	LEP	BT020625	FOR	tgcgctgtggacccctgtatc	152
			REV	gacggactgcgtgtgtgag	
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	BC102589	FOR	gccgtaacttctgtgctgtgcc	226
			REV	tctctgccttgactgtgccg	

[1]FOR = Forward primer, REV = Reverse primer

mRNA and histochemical analysis. At BP2 and BP3, 8 cm or 8 mm skeletal muscle samples were taken by needle biopsy or shot biopsy respectively for m RNA and histochemical analysis. Samples were immediately frozen in liquid nitrogen and stored at -80°C.

Total RNA extraction and cDNA synthesis: Total RNA was extracted from muscle tissues using the ISOGEN (NIPPON GENE) system according to the manufacturer's protocol. To quantify the amount of total RNA extracted, absorbance was measured with a calculator (Amersham GeneQuant RNA/DNA Calculator) for each sample. RNA purity was calculated using the 260/280 absorbance ratio and ratios confirmed to be between 1.90 and 2.00.

The first strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using ReverTra Ace (TOYOBO) reverse transcriptase with random primer (TOYOBO). The synthesized cDNAs were stored at -40°C.

Quantitative real-time PCR: After reverse transcription analysis of expression of the genes encoding peroxisome proliferator-activated receptor gamma 2 (PPARγ2), CCAAT/enhancer-binding protein alpha (C/EBPα), Stearoyl-CoA Desaturase (SCD), Glucose-6-Phosphate Dehydrogenase (G6PD), Fatty Acid Synthase (FASN) and Leptin was performed by real-time RCR using a Line Gene system (BioFlux). The first strand cDNA was diluted with RNase- and DNase-free water and amplified using SYBR Green Real-time RCR Master Mix (TOYOBO) with gene-specific primers (Table 2). The real-time PCR reaction was initially carried out for 1 min at 95°C, followed by 42 cycles of 15 s at 95°C, 15 s at 59°C and 30 s at 72°C with a Melting Curve analysis, 2 min at 95°C and 1 min at 65°C. The housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control. Expression levels for each gene were calculated relative to GAPDH mRNA expression. Reactions were performed twice for each sample.

Histochemical analysis and fat content: Serial frozen sections (8 μm in thickness) were stained with oil-red O and HE stain to detect adipocytes and measure adipocyte diameter.

Statistical analysis: In this study, the data at BP1 and BP2 was analyzed between group H (n = 7) and group N plus group R (n = 14) because the nutritional treatment in group N and group R was the same until BP2. The mRNA expression was analyzed by Analysis of Variance (ANOVA) using the Statistica program. Differences were considered significant at $p<0.05$.

This experiment was carried out under the guidelines for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and the Notification (No. 6) of the Government.

RESULTS AND DISCUSSION

The nutritional level of feed is one of the most important factors in determining growth, quality and quantity of products in domestic animals. Intramuscular fat deposition is associated with genetic background, as well as the development (mature body size) and nutritional level of an animal (Pethick *et al*., 2004). Wagyu cattle (Japanese Black) are genetically predisposed to deposit intramuscular fat. In the study, gene expression analysis of LT from F1 was carried out in the early developmental stage.

Performance of experimental animals and biopsy time points were shown in Table 1. The difference in body weight at BP2 was 1.2-fold between groups and this was significantly different ($p<0.05$). Similarly, at BP3 the body weight was significantly different among each group ($p<0.01$) (Table 1).

No adipocytes were detected in samples at BP1. Subsequently, adipocytes were detected at BP2 but there were no significant differences in adipocyte diameter

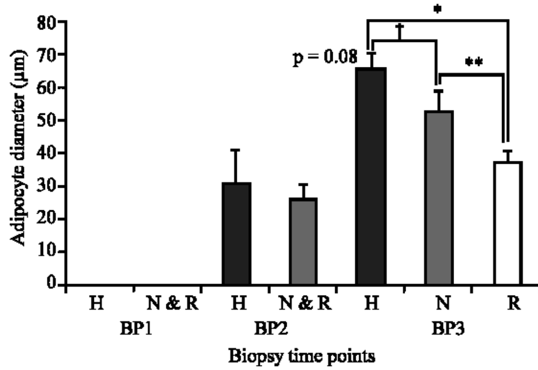

Fig. 1: Diameters of adipocytes in *longissimus* muscle of cattle subjected to nutritional treatments of high energy group (H), normal energy group (N) or roughage group (R). Regarding biopsy time points, the beginning of different milk reatment (45 days of age), the end of different milk replacer treatment (4 months of age) and the end point of different nutritional treatment (10 months of age) are indicated by BP1, BP2 and BP3, respectively. Groups N and R were treated under the same conditions from BP1-BP2 therefore, the two groups were combined until BP2 (N&R). There were no detectable adipocytes in samples at BP1. Results are means±SEM. *$p<0.01$, **$p<0.05$, §$p<0.1$

between group N and R and H. On the other hand, adipocyte diameters were significantly different between group H and group R (group H/group R = 1.82 in LT; $p<0.01$) and between group N and group R (group N/group R = 1.45 in LT; $p<0.05$) at BP3. The diameter in group H was larger than that in group N (group H/group N = 1.25; p = 0.08) (Fig. 1). In summary for BP3, the adipocyte diameters were largest in group H, second largest in group N and smallest in group R, when cattle were gently given a different feeding regime. Bartlett *et al.* (2006) demonstrated that body weight gain can be markedly improved by increasing feeding rate and by increasing the amount of protein in milk without fattening.

In the similar study, intensive nursing with high protein and a low fat milk replacer markedly impacted on the development of frame size (bones etc) until BP2. On the other hand, it had no impact on the composition of muscle fiber type and the size of muscle fibers (Inada *et al.*, 2010). It was suggested that skeletal muscles enlarge rather than become thicker as the frame grows until BP2. From the present results, similarly we inferred that intensive nursing did not have a great effect on intramuscular adipogenesis at BP2 (weaning point).

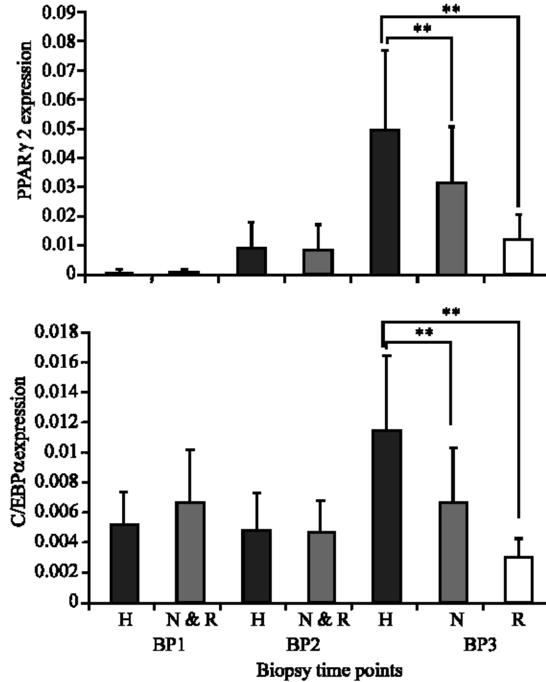

Fig. 2: Comparison of peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and CCAAT/ enhancer-binding protein alpha (C/EBPα) gene expression patterns among the high energy group (H), normal energy group (N) and roughage group (R) by real-time PCR analysis. Regarding biopsy time points, the beginning of different milk replacer treatment (45 days of age) the end of different milk replacer treatment (4 months of age) and the end point of different nutritional treatment (10 months of age) are indicated by BP1, BP2 and BP3 respectively. For each gene, the expression level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), measured at the same time. Results are means±SD. **$p<0.05$

In order to validate gene expression levels of PPARγ2, C/EBPα, SCD, G6PD, FASN and Leptin, real-time PCR assays were carried out using muscle samples taken from crossbred LT muscles. Figure 2-4 show the mRNA expression for six genes at three biopsy time points (BP1-BP3). Expression for all genes in the BP1 and BP2 samples showed no significant differences (Fig. 2-4). In contrast, there were significant differences in the BP3 samples.

PPARγ2 gene expression in group H at BP3 was greater ($p<0.05$) than in group R, while C/EBPα gene expression in group H at BP3 was greater than in group N ($p<0.05$) and group R ($p<0.01$). The SCD, G6PD, FASN and

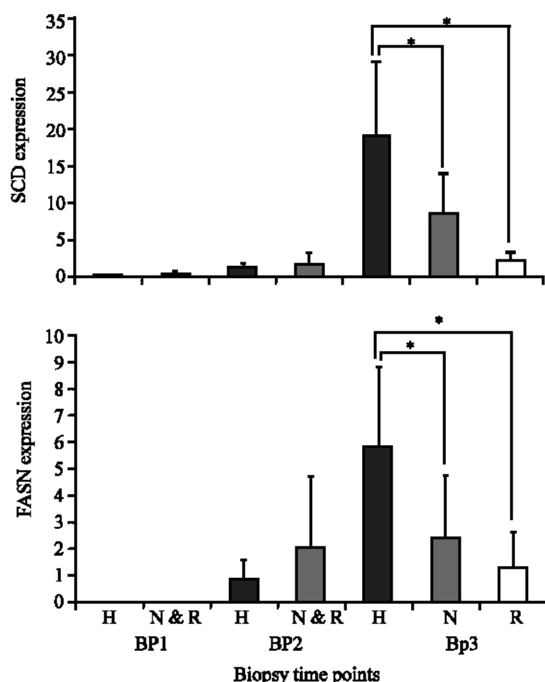

Fig. 3: Comparison of Stearoyl-CoA Desaturase (SCD) and Fatty Acid Synthase (FASN) gene expression patterns among the high energy group (H), normal energy group (N) and roughage group (R), by real-time PCR analysis. Regarding biopsy time points, the beginning of different milk replacer treatment (45 days of age), the end of different milk replacer treatment (4 months of age) and the end point of different nutritional treatment (10 months of age) are indicated by BP1, BP2 and BP3, respectively. For each gene, the expression level was normalized to that of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), measured at the same time. Results are means±SEM *p<0.01

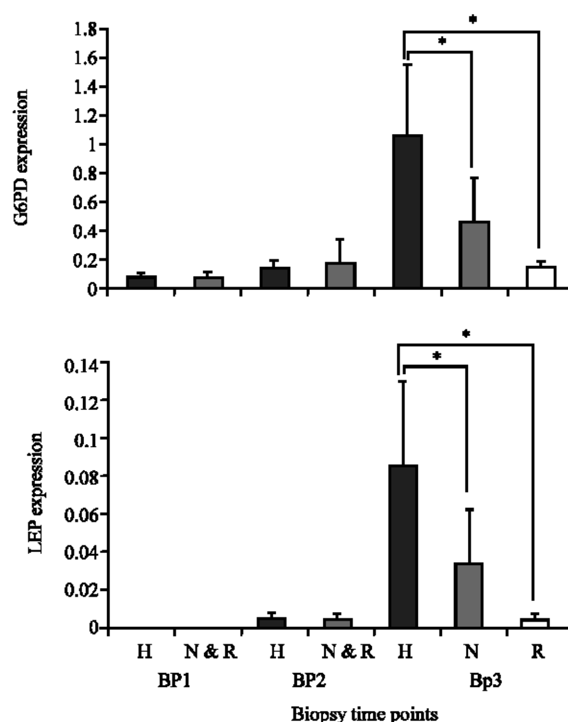

Fig. 4: Comparison of Glucose-6-Phosphate Dehydrogenase (G6PD) and Leptin (LEP) gene expression patterns among the high energy group (H), normal energy group (N) and roughage group (R) by real-time PCR analysis. Regarding biopsy time points, the beginning of different milk replacer treatment (45 days of age), the end of different milk replacer treatment (4 months of age) and the end point of different nutritional treatment (10 months of age) are indicated by BP1, BP2 and BP3, respectively. For each gene, the expression level was normalized to that of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), measured at the same time. Results are means±SD. *p<0.01

Leptin gene expressions in group H at BP3 were greater ($p<0.01$) than in group N and group R. Comparing the expression levels between group N and group R at BP3, the expression levels were significantly different except for FASN ($p<0.01$) (Fig. 2-4). During adipocyte differentiation, transcription of genes encoding the adipogenic transcription factors PPAR and C/EBP are important (Hausman *et al*., 2009). The appearance of PPARγ and C/EBPα ensures the maintenance of the differentiated state of the adipocyte andthe two transcription factors reciprocally regulate each other (Rosen, 2005). The expression of PPAR gamma 2 (PPARγ2) is tissue-specific; PPARγ2 is predominantly expressed in adipose tissue (Tontonoz *et al*., 1995; Mueller *et al*., 2002). Saladin *et al.* (1999) hypothesized that PPARγ2 initiates adipocyte differentiation. It has also been reported that PPAR gene expression was induced by nutritional treatments. In this study, the expression levels of PPARγ2 and C/EBPα showed no significant differences at BP1 and BP2 but there were significant differences in group H relative to those of group N and R at BP3 (10 months of age) (Fig. 2).

These results suggested that higher levels of nutrition from 5-10 months of age significantly influence expression of the genes related to adipocyte differentiation in LT. SCD is an enzyme that catalyzes the Δ9 desaturation of saturated fatty acids to Monounsaturated Fatty Acids (MUFAs). In cattle, fat containing abundant unsaturated fatty acids has a

lower melting point (Yang *et al*., 1999) and the fatty acids contribute to the softness of bovine fat (Melton *et al*., 1982). The unsaturated fatty acids are generally regarded as being beneficial for human health (Scollan *et al*., 2006). Thus, bovine SCD is related not only to health benefits but also to fat quality in beef. In the study, SCD gene expression was significantly higher in group H at BP3 (Fig. 3). In this regard, the differences in nutrition in the early developmental stage could affect meat quality and especially fat quality in LT of F1.

Furthermore, FASN gene expression was also higher in group H than other groups at BP3 (Fig. 3). FASN, which is a multifunctional enzyme with a central role in the metabolism of lipids is involved in *de novo* fatty acid synthesis. With this in mind in this research, the feeding of a high nutrition TMR diet from 5-10 month of age would have a major impact on fat metabolism in skeletal muscle.

The binding sequences of transcriptional factors such as C/EBPα are conserved in promoter sequences of mouse SCD1 and human SCD (Bene *et al*., 2001). In addition, the promoter sequence of bovine SCD is highly homologous to that of mouse and human (Keating *et al*., 2005), suggesting that C/EBPα is also an important transcriptional factor of bovine SCD. In this study, the gene expression levels of C/EBPα and SCD were significantly higher at BP3 (Fig. 2 and 3). At BP3, the SCD gene expression level in group H was approximately two- and ten times-higher than that in group N and group G, respectively. These large differences in SCD gene expression might relate to the differences in C/EBPα gene expression levels.

Expression of the G6PD gene was significantly higher in group H at BP3 than that of group N and group G (Fig. 4). G6PD catalyzes the oxidative reaction of the pentose cycle and Martin and Makula (1987) have shown that pentose cycle oxidative activity is closely related to rates of lipogenesis. Subsequently, Belk *et al*. (1997) reported a putative involvement of G6PD activity in the marbling of the oxido-glycolytic LT from Wagyu and Angus steers. Thus, G6PD is an important factor in adiposity and lipogenesis.

The expression of the Leptin gene was significantly higher in group H at BP3 than that in group N and group G (Fig. 4). Serum concentrations of leptin were significantly associated with carcass composition (marbling and back fat depth) and quality grade in crossbred cattle (Geary *et al*., 2003). A Leptin hormone was found to be mainly synthesized in adipose tissue at a rate strongly related to adiposity in ruminants (Chilliard *et al*., 2005). Adipocyte size may influence leptin synthesis and secretion, because larger adipocytes contained more leptin mRNA (Auwerx and Staels, 1998). These results were also supported by data showing that the fat cell diameter was the greatest and expression of the Leptin gene was the highest, in group H at BP3.

Furthermore, Bonnet *et al*. (2007) reported that G6PD and Leptin are closely related to the deposition of intramuscular adipose tissue in beef cattle. The results showed that the expression levels of G6PD and Leptin were relatively high, so the adiposity and lipogenesis in skeletal muscle were changed by altered nutrition levels during the 5-10 months period.

In this study, calves fed intensified milk replacer showed superior performance (frame size and weight gain) to those under other treatments and following this high nutritional feeding accentuated the adipogenesis. Finally, there was a substantial difference in body weight at BP3. These results may have an influence on later development, meat quality and quantity. The change of body weight from BP2 to BP3 was about two-fold, irrespective of nutritional level in all groups.

On the other hand, there were marked differences in the expression of genes related to adipogenesis and the development of adipocytes in group H. Therefore, these results may be affected not only by the nutritional level during post-weaning but also by intensive nursing.

CONCLUSION

The feed quality during the early growth stage influences the mRNA expression of genes related to adipogenesis and fatty acid synthesis in skeletal muscle. The growth performance was remarkably different between groups. Therefore, the nutritional level from 5-10 months of age would markedly alter intramuscular adipogenesis, indicating different expression levels of adipogenesis-related genes in LT of F1. However, further research is needed to investigate the effects of rapid early growth rate and intramuscular adipogenesis on body composition and subsequent productivity.

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