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Running Title	Effects of increased protein intake in pregnant cow
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ACCEPTED

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Abstract

Understanding adipocyte development in fetus during bovine pregnancy is important for strengthening fattening technology. Additionally, nutritional level of dams during pregnancy has the potential to improve offspring growth and fat development. The purpose of this study is to evaluate the intramuscular adipocyte development and expression level of related genes in bovine fetus, and the effect of increased crude protein (CP) intake during pregnancy on the growth performance and carcass characteristics of male offspring. Eighty six pregnant Hanwoo cows (average body weight, 551.5 ± 51.3 kg, age 5.29 ± 0.61 y) were used. Fetuses were collected at 90, 180 and 270 d of gestation from 18 pregnant Hanwoo cows. The remaining 68 pregnant cows were randomly assigned to 2 feeding groups. The control (CON) group was provided the standard protein diet ($n = 34$), and treatment (TRT) group was provided a diet with a 5% increase in CP intake ($n = 34$). Male offspring were divided into two groups according to protein treatment of the pregnant cows: CON male offspring (CON-O) and TRT male offspring (TRT-O). Intramuscular adipocytes were found in the fetal skeletal muscle after 180 days of gestation. Male calf's birth weight increased in the TRT group compared to that in the CON group ($p < 0.002$). The final body weight ($p < 0.003$) and average daily gain ($p < 0.019$) of male offspring were significantly higher in TRT-O than in CON-O. The feed conversion ratio was also improved by 10.5% in TRT-O compared to that in CON-O ($p < 0.026$). Carcass weight was significantly higher in the TRT-O group than that in the CON-O group ($p < 0.003$), and back fat was thicker in the TRT-O group ($p = 0.07$). The gross receipts and net income were higher in TRT-O than in CON-O ($p < 0.04$). Thus, fetal intramuscular fat can be formed from the mid-gestation period, and increased CP intake during pregnancy can increase net income by improving the growth and carcass weight of male offspring rather than intramuscular fat.

Keyword : Fetal, Intramuscular adipocyte, Hanwoo, crude protein intake, Net income

INTRODUCTION

Intramuscular fat (marbling) is the most important factor determining the beef quality and directly affects profitability. Various methods have been investigated, and substantial efforts have been made to efficiently increase intramuscular adipogenesis [1]. Most of the previous studies have focused on the fattening period. However, maternal nutrition during gestation has recently been shown to affect offspring growth and intramuscular fat [2, 3]. Consequently, interest in fetal programming has increased. It is important to understand fetal growth patterns throughout gestation to apply fetal programming effectively. Fetal development in ruminants shows patterns of organ formation in early gestation, myogenesis in mid-gestation, and adipogenesis in late gestation [4]. However, few studies have assessed gene expression and the development of intramuscular adipocytes in fetuses during gestation.

Controlling the nutritional level of pregnant cows is one of the most useful strategies to maximize offspring performance. The level of nutrition consumed by the mother during gestation plays an important role in fetal growth and development [5]. It also affects the acquired growth and physiological function of offspring, which can change the productivity and quality of livestock [6]. Although many nutrients are present in the feed, a single nutrient can affect fetal programming [7]. Proteins are essential nutrients for the production of enzymes and hormones, as well as for cell proliferation and differentiation. Recent studies have shown that high-protein grass or concentrate intake in pregnant cows improves fat storage in the offspring [7, 8]. These results suggest that the protein may positively affect intramuscular adipocyte differentiation in the fetus. However, excess protein during gestation period induces oxidative stress in the fetus [9] and reduces insulin-like growth factor-I levels in the maternal

blood, affecting the distribution of nutrients between the dam and fetus [10]. One important commonality is that excessive maternal nutrient supplementation reduces the birth weight of offspring and impedes their growth [9, 11, 12]. To avoid this problem, it was necessary to set an appropriate increment range.

We hypothesized that potential intramuscular fat can be formed during the fetal period and that increase of crude protein (CP) intake during pregnancy could increase economic profitability through improved growth and meat quality (marbling) of male offspring without negative impact. Therefore, the first objective of this study was to investigate the growth, intramuscular adipogenesis, and expression of major genes in fetuses during gestation. The second objective was to investigate the effect of increase in CP intake during pregnancy on body weight (BW) and subsequent reproduction in the mother and evaluate the productivity and economics of male offspring born from them.

Materials and Methods

Animals, experimental design, and management

Eighty six multiparous Hanwoo cows (average weight 551.5 ± 51.3 kg, age 5.29 ± 0.61 y, parity 3.52 ± 0.23) were used in the present study. Hanwoo cows underwent artificial insemination (AI) after estrus synchronization, and pregnancy was detected using rectal ultrasound 60 days after AI. Eighteen pregnant cows were randomly selected to investigate intramuscular fat formation in fetal. Cows were allocated 6 per group and housed 3 per pen (5×10 m), and were fed according to the standard nutrition requirement until slaughter at 90, 180, and 270 d of gestation. To determine the effect on increased CP intake during gestation, 68 pregnant cows were randomly assigned into 2 groups with different feeding levels. The control group (CON) was assigned 34 pregnant cows and provided standard protein

requirements [13] (Concentrate I: 3.54 kg + rice straw: 4.75 kg). The remaining 34 pregnant cows were assigned to the treatment group (TRT), and high-protein concentrate (II) was provided at a ratio of 3:1 to normally concentrate (Concentrate I: 2.66 kg + concentrate II: 0.88 kg + rice straw: 4.75 kg). Cows were maintained 4 per pen (5×10 m), and concentrate and rice straw were provided twice a day. They were moved to the delivery room 2 months before delivery and managed individually. The ingredients and chemical composition of the experimental diets are listed in Table 1.

Only male offspring were used to evaluate the fetal programming effect on increased CP intake during pregnancy. After birth, male calves were wiped with a clean towel, and their belly buttons disinfected. The calves were kept in the delivery room with the mother until weaning, and a small amount of starter and grass was provided. Male calves were weaned at 90 d of age and divided into 2 groups according to the protein levels of the pregnant cows: CON male offspring (CON-O) and TRT male offspring (TRT-O). Male calves were allocated 4 or 5 per pen (5×10 m) with sawdust to a thickness of approximately 20 cm and surgically castrated at 6 months of age.

The feeding method was divided into growing period and fattening period. Timothy hay was fed in the growing period and rice straw was used in the fattening period. The timothy hay was started with 1.0 kg at birth and increased by 1 kg every month to 4 kg at 7 months of age, and was fixed at 4.5 kg for 8 to 13 months. From 14 months Rice straw was reduced by 0.5 kg every 2 months from 3 kg, and was fixed at 1 kg from 22 months of age until slaughter. Concentrate feed was started to provided 1.5kg at birth, and the amounts was increased by 0.5kg every month to 6.5kg at 13 months of age. At the fattening period, concentrate feed was provided 7.0kg at 14 months of age, and the amounts was increased by 0.5kg per months up to a maximum of 10kg, and fixed at 9kg from 4 months before slaughter. Feed was provided twice

daily, and water and mineral blocks were freely available. The ingredients and chemical compositions of the experimental diets are listed in Table 2.

Tissue sample collection

Pregnant cows were slaughtered at different gestation periods (90, 180, and 270 days). Immediately after slaughter, the fetus was removed from the uterus. The weight and length of the fetuses were measured using an electronic scale and tape measure. The skin and subcutaneous tissues of the fetal *longissimus dorsi* (LD) and *semimembranosus* (SM) were removed, and pure skeletal muscle samples were collected. Skeletal muscle samples were divided into two sterile tubes: one was frozen in liquid nitrogen until messenger ribonucleic acid (mRNA) analysis, and the other was fixed with 10% neutral buffered formalin for use in histological analysis.

Tissue sections and staining

The LD and SM samples were trimmed so that the cross-section could be inspected, and washed in running water for 4 h. For dehydration of the tissue sample, 10%, 20%, and 30% sucrose solutions were substituted, and then the substituted tissue was put into the optimal cutting temperature (OCT) compound, and the tissue was quickly frozen and embedded. After freezing, each sample was sliced into 10 μ m slides using a freezing microtome, and the sliced tissues were mounted on silane-coated glass slides. Tissue slides were washed with phosphate buffer saline (PBS). 5 mL of PBS with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) solution was added to the slide, and incubated at 20°C for 30 min. Tissue slides were washed and blocked with 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and PBST (22.52 mg/mL glycine in PBS + 0.1% Tween 20). After blocking, the samples were incubated

with primary antibodies against myosin light chain 2 (Abcam, Cambridge, UK) and body-fit solution (4, 4 - Difluoro - 1,3,5,7,8 - Pentamethyl - 4 - Bora - 3a, 4a - Diaza - s - Indacene) for 1 h in the dark environment at 20°C. After washing with high-salt PBS, the samples were incubated with a secondary anti-body solution. Finally, the slides were coated with glycerin, and the slides were covered with a cover glass and observed under an optical microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan).

mRNA extraction and qPCR analysis

2 g Sample and 1ml TRIzol (Ambion, Carlsbad, CA, USA) were put into a screw tube and homogenized with a homogenizer (TissueLyser 2; Qiagen, Hilden, Germany). 100 uL of chloroform was added to the screw tube and vortexed, and centrifuge at $17,000 \times g$ for 15 min (4°C). Supernatant 1ml was transferred to a new microtube, and isopropyl alcohol was added, followed by mixing. After centrifuging the microtube at $17,000 \times g$ for 15 min (4°C), the supernatant was removed. Again, after adding 1 mL of 75% ethanol and centrifugation at $10,600 \times g$ for 5 min (4°C), the supernatant was removed. RNA was dissolved by adding in diethyl pyrocarbonate to the microtube, and synthesized using a cDNA synthesis kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover; TOYOBO, Osaka, Japan).

The gene expression level was analyzed using performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The myogenic markers used were myogenin (MyoG), myosin heavy chain 1 (MYH 1), and myosin heavy chain 2A (MYH 2A), and the adipogenic markers used were CCAAT/enhancer binding protein β (C/EBP β), peroxisome proliferator activated receptor γ (PPAR γ), and stearoyl-CoA desaturase (SCD). The primer sets used are listed in Table 3. The reaction mix was 5.5 μ L of distilled water, 2 μ L of cDNA, 1 μ L each of forward and reverse primers, 0.5 μ L of the probe, and 10 μ L of the

master mix. The reaction conditions were as follows: 40 cycles of 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C. The housekeeping gene 40S ribosomal protein S9 (RPS9) served as an internal control to normalize the relative mRNA expression.

Growth and reproductive performance

The BW of the animals was measured using a cattle weight bridge installed in the barn. The average daily gain (ADG) was calculated based on the BW difference and the number of days of feeding. Dry matter intake (DMI) was calculated by measuring the quantity of residual feed per pen before feeding in the morning, and the feed conversion ratio (FCR) was calculated based on DMI and ADG values.

Calving cows were subjected to AI from the second estrus (approximately 21 d after the first estrus) and service per conception (SPC), defined as the number of service inseminations performed to produce a conception. The conception rate was calculated as the proportion of pregnancies after the first AI.

Carcass traits

Male offspring were slaughtered in a slaughterhouse at 30 months. After the carcass was chilled in a refrigerator at 4°C for 24 h, carcass weight was measured. Next, the left side of each carcass was cut between the thirteenth rib and the first lumbar vertebra to determine meat yield and quality. The carcasses were evaluated according to the Korean carcass grading system [14]. Yield grades were evaluated as A, B, and C, and quality grade were evaluated as 1++, 1+, 1, 2, and 3. The auction price was determined as the winning bid price according to yield and quality grades

Economic analysis

A partial budget analysis was conducted to evaluate the economic ramifications. Gross receipts were determined based on the selling price per carcass and product of the male offspring. The operating costs were calculated by adding the feed costs (including commercial concentrate and roughage costs) and other costs (water, power, fuel, veterinary and medicine, automobile, farm implementation, farm building and facilities, miscellaneous materials, interest on borrowed capital, land rent, labor, excretion disposal, and production management costs), as provided by the Livestock Production Cost Survey [15]. Net income was calculated as gross receipts minus the operating costs.

Statistical analysis

All data were analyzed using SAS package 9.1 software program (SAS Institute Inc., Cary, NC, USA). One-way ANOVA was used for weight (kg), length (cm), intramuscular adipocyte area (%), and gene expression (arbitrary units) in the fetus by the day of pregnancy (90 d, 180 d and 270 d). If there was a significant difference between treatment groups, further analysis was performed by Tukey's post hoc test. Data from the mothers (CON or TRT) and male offspring (CON-O or TRT-O) based on increased CP intake were analyzed using t-tests. Data are presented as the mean and SEM. Differences between treatments were considered significant at $P < 0.05$.

RESULTS

Fetal growth

Changes in fetal BW and length throughout the pregnancy period of Hanwoo cattle are shown in Table 4. Fetal BW continually increased with the progression of the pregnancy period ($p <$

0.01); in particular, it increased rapidly between 180 and 270 d of gestation. Fetal length increased by more than 3 times to 84.0 cm at 270 d compared to 25.5 cm at 90 d ($p < 0.01$).

Fetal intramuscular adipogenesis

The results of the immunofluorescence staining and adipocyte area in the fetal skeletal muscle throughout the pregnancy period of Hanwoo cattle are shown in Figure 1. Intramuscular adipocytes were observed around the blood vessels after 180 d of fetal growth, and the size of the muscle fibers increased continuously. The adipocyte area increased at 270 d compared to that at 180 d in both LD and SM. In addition, the adipocyte area was similar between the LD and SM muscles at 180 d but was higher in the LD muscle than in the SM at 270 d.

Expression of genes related to adipogenesis and myogenesis

Changes in the mRNA expression of genes related to adipogenesis and myogenesis in fetal skeletal muscle throughout the pregnancy period of Hanwoo cattle are shown in Figure 2. The mRNA expression of MyoG in LD muscle was significantly decreased at 270 d compared to 180 d of gestation ($p < 0.05$). MYH1 expression was not affected during gestation, but MYH2A expression was significantly increased at 180 d of gestation ($p < 0.05$). The mRNA expression of MyoG in SM muscle did not change throughout the pregnancy period, but MYH1 expression was significantly higher at 270 d of gestation ($p < 0.05$), and MYH2A expression increased throughout the pregnancy period, but not significantly different. The mRNA expression of SCD was greater at 180 d than at 90 and 270 d in LD muscle ($p < 0.05$). Similarly, the mRNA expression of PPAR γ was also the highest at 180 d ($p < 0.05$). C/EBP β mRNA expression increased significantly at 180 and 270 d from 90 d of gestation ($p < 0.05$). No differences were observed in the mRNA expression of SCD and PPAR γ in SM muscle, but the mRNA expression

of C/EBP β was affected by the pregnancy period ($p < 0.05$).

Cow BW, subsequent reproduction, and calf birth BW

The effects of increased CP intake during pregnancy on growth performance, birth BW, and subsequent reproduction in Hanwoo cattle are shown in Table 5. Protein supplementation did not affect BW, ADG, and DMI of Hanwoo cows during the pregnancy period. The birth BW of female calves was higher in the TRT group than in the CON group ($p < 0.01$). Similarly, the birth BW of male offspring born in TRT was higher than of those born in CON ($p < 0.01$). SPC and conception rates were similar between the treatments.

Growth performance of male offspring

The effects of increased CP intake during pregnancy on the growth performance of the male offspring of Hanwoo cattle are shown in Table 6. The initial BW during the growing period was higher in TRT-O than in CON-O, and the final BW during the growing period was higher in TRT-O than in CON-O ($p < 0.04$). However, there were no differences in the ADG, DMI, and FCR. The finishing period was, on average, 91.1 kg higher in TRT-O compared to CON-O ($p < 0.01$). ADG was 17.3% higher in TRT-O compared to CON-O. Although the DMI was statistically significant, the numerical difference was small, and the FCR was 16.4% lower in TRT-O than in CON-O, but the difference was not significant. During the entire test period, the average ADG was higher in the TRT-O group than in the CON-O group ($p < 0.02$), and the FCR was also improved ($p < 0.03$).

Carcass traits of male offspring

The effects of increased CP intake during pregnancy on carcass traits of male offspring of

Hanwoo cattle are shown in Table 7. Carcass weight increased by 57.3 kg in TRT-O compared to CON-O ($p < 0.01$), and back fat thickness also increased in TRT-O. The rib eye area was similar between the treatments, and the yield index ($p < 0.02$) and yield grade score ($p < 0.02$) were higher in CON-O than in TRT-O. The marbling score was 14.2% higher in TRT-O than in CON-O, but there was no significant difference. Meat color, fat color, texture, maturity, quality grade score, and auction cost also showed little difference between the treatments.

Economic efficacy

The effects of increased CP intake during pregnancy on economic efficacy in Hanwoo cattle are shown in Table 8. Gross receipts were significantly increased in TRT-O compared with CON-O ($p < 0.04$), and feed costs also showed a significant increase in TRT-O, but the numerical difference was not significant ($p < 0.01$). Net income increased by an average of 1,331 USD/head in TRT-O compared with CON-O ($p < 0.04$).

DISCUSSION

Fetal growth

Fetal growth depends on nutrients received during pregnancy. In cattle, fetal organogenesis simultaneously with placental development. A heartbeat can be detected within at least 21 days, and the extremities begin to develop at 25 d of pregnancy, followed by the development of vital organs, including the liver, lungs, brain, and kidneys. In addition, Chalk [4] reported that the development of ovaries and testes begins at 60 d of age. Mao et al. [16] reported that significant kidney growth occurs during mid-pregnancy, and accelerated liver and heart growth occurs during late pregnancy. In ruminants, most fetal structural growth occurs in the last third of the pregnancy period [17], where important organs and tissues are established within a few months,

while 75% of fetal growth occurs 2 months before delivery [18]. Mao et al. [16] found that the weight of the semitendinosus muscle of the fetus increased from 214- to 483-times at 9 months compared to that at 3 months of pregnancy, depending on the breed. The fat-weight to body-weight ratio and the total body fat increased continuously throughout the pregnancy period. Ferrell et al. [19] also reported that cattle fetuses undergo rapid growth during the middle to late stages of pregnancy. Similar to the findings of previous studies, our results showed that Hanwoo cattle fetuses grew rapidly between 180 and 270 d of pregnancy and remarkably during the late stages of pregnancy. Because the vital organs for maintaining the life of the fetus during early pregnancy are relatively unaffected by maternal nutritional status [20, 21], overall growth is presumed to be dominant in the late stage of pregnancy because vital organs are a higher priority for nutrient distribution than skeletal muscle and fat in early pregnancy [22]. Because the growth rate of the fetus increases after the middle stages of pregnancy, the mother requires additional nutritional supplementation to supply sufficient nutrients to the fetus.

Fetal muscle development

The highest developmental periods of organogenesis, myogenesis, and adipogenesis in the fetus differ throughout gestation [7, 16, 23]. Fetal muscle development involves 2 processes: an increase in muscle cell numbers (hyperplasia) and their size or diameter (area and length), known as hypertrophy [24]. Primary myogenesis occurs during early pregnancy with minimal skeletal muscle development. Secondary myogenesis occurs during mid-pregnancy, and skeletal muscles develop in earnest [4]. The total number of muscle cells in the fetus is fixed by 240 days of pregnancy, and the area and length of the muscle increase in the second half of the pregnancy period [25]. The number, type, and size of muscle fibers are the main factors that determine muscle mass, and the number of muscle fibers is positively correlated with the

potential for growth after delivery [26].

Myogenesis is a complex process involving the proliferation and differentiation of precursor cells and is affected by various genes and myogenic regulatory factors, including MyoG [22]. MyoG is an important transcription factor involved in stimulating muscle growth by inducing myogenesis and contributing to the formation of muscle cells [27]. In addition, MyoG is directly involved in muscle growth (muscle hypertrophy) and affects carcass weight [28, 29]. Myosin heavy chain is involved in muscle contraction and force generation [30]. Four isoforms have been reported in mammalian skeletal muscles [31]. MYH 1, which has low ATPase activity and slow contraction action, is necessary for posture maintenance and is mainly found in the red muscles [31]. MYH 2A is abundant in constantly used muscles and is highly metabolic due to oxidation and glycolysis processes.

In this study, the fetal muscle cell size increased throughout the pregnancy period, and the expression of genes (MYH 1, MYH 2A) related to muscle contraction (motility) was higher than that of muscle cell differentiation (MyoG). Similar to our findings, Duarte et al. [1] found no difference in the mRNA expression of MyoG in the skeletal muscle of the fetus during the gestation period (150 to 240 d). Picard and Gagaoua [24] reported that metabolic and contractile maturation of muscle fibers mainly occurs in the last trimester of pregnancy. Therefore, muscle generation is dominant from the beginning to the middle stages of pregnancy, and metabolic processes related to muscle hypertrophy and contraction are actively generated after the middle stage of pregnancy.

Fetal intramuscular adipogenesis

Adipose tissue is vital for energy storage, systemic metabolism, homeostasis regulation, and endocrine and paracrine signaling. Adipose tissue is formed during the prenatal period and

develops during the late stages of pregnancy. Du et al. [7] reported that fetal adipocytes were produced before and after 4 months of gestation and lasted up to 8 months after delivery. Chalk [4] also reported that myogenesis and adipogenesis are derived from the same mesenchymal stem cells and that more mesenchymal stem cells are used for adipogenesis in the later stages of pregnancy. In a study by Mao et al. [16], fetal body fat increased continuously from 3 to 9 months of pregnancy, resulting in a significant increase in the total fat to body weight ratio.

Adipocytes grow by receiving nutrients and oxygen from blood vessels and are surrounded by an extensive capillary network [32, 33]. As the formation of new blood vessels is required to grow and develop adipose tissue, angiogenesis occurs in adipose tissue [34, 35]. Capillary endothelial cells interact with fat cells through paracrine signaling pathways, extracellular components, and direct cell-cell interactions [32, 36, 37]. In addition, activated adipocytes produce angiogenic factors such as vascular epidermal growth factor, fibroblast growth factor 2, leptin, and hepatocyte growth factor, which, alone or in cooperation, increase angiogenesis and promote the growth and metabolism of adipose tissue [38]. Consequently, the growth and development of adipose tissue are highly dependent on angiogenesis. In this study, the formation of adipocytes around blood vessels in fetal skeletal muscle was observed at 180 d of gestation. Therefore, intramuscular adipocytes in the fetus can be formed in the middle stage of pregnancy, similar to other adipose tissues. It is assumed that their formation around the blood vessels increases interaction with blood vessels to obtain a supply of nutrients.

Differentiation of mature adipocytes is regulated by transcription factors such as the C/EBP family and PPAR γ [38]. C/EBP β not only regulates the expression and activity of PPAR γ but is also involved in the synthesis of PPAR γ ligands [40]. Adipocyte differentiation is also caused by stem cells present in the muscle [41], and the proliferation, differentiation, and size of adipocytes in the muscle are closely related to the marbling score [41, 42].

Mitochondria use energy and fatty acids to form acetyl-CoA, which is used as a precursor for intramuscular fat synthesis [43], and acetyl-CoA synthesizes long-chain fatty acids (saturated fats) through the action of various enzymes. SCD converts saturated fatty acids into monounsaturated fatty acids, allowing fat accumulation [44]. In this study, the expression of SCD and PPAR γ was high in LD muscle at 180 d of gestation, and the expression of C/EBP β continuously increased throughout the pregnancy period. Similarly, in a study by Duarte et al. [1], the mRNA expression of PPAR γ in fetal skeletal muscle was higher at 190 d of gestation than at 135 and 240 d of gestation. In addition, the timing at which the expression of these genes increased and the timing of intramuscular adipocyte discovery coincided. Therefore, SCD, PPAR γ , and C/EBP β are important genes related to the differentiation of intramuscular fat during the prenatal stage. Since the expression of C/EBP β in fetal skeletal muscle and the area of intramuscular adipocytes continuously increased, it was determined that the differentiation of intramuscular adipocytes was maintained from the middle stages of pregnancy until delivery.

Cow BW and subsequent reproduction

Nutrients consumed by pregnant cows are vital for maternal maintenance and growth, fetal development, and postpartum recovery. In this study, increased CP intake did not affect pre- or postpartum cow weight or subsequent fertility (Table 5). Wilson et al. [45] reported that there was no difference between pre- and postpartum (115 d) BW, AI conception, and overall pregnancy in a 129% treatment group compared to the 100% protein requirement group. Some studies did not show an effect on pre- and postpartum BW and subsequent reproduction according to protein supplementation for a period of time [46, 47, 48]. However, in a study by Valiente et al. [49], cow BW and ADG were higher in heifers with HP (121% of protein

requirements) than in LP (64% of protein requirements) without any difference in subsequent reproduction. Funston et al. [50] and Martin et al. [51] reported similar results. Unlike previous studies using cows, heifers continue to grow physiologically, so it is judged that protein supplementation during pregnancy can affect body growth.

Excessive increased CP intake in cows increased the blood urea nitrogen concentration, negatively affected the uterine environment, and reduced reproductive function [52]. Since there was no difference in SPC and conception rates between treatments in this study, it is speculated that increase 5% CP intake does not adversely affect subsequent reproduction. Similar to our study, Stalker et al. [48] found that protein supplementation (0.45 kg/cow per d, protein 42%) during pregnancy did not affect cows' subsequent reproductive performance and fertility rates.

Growth performance and carcass traits of male offspring

Controlling maternal dietary protein intake during pregnancy is essential for embryo survival, growth, and development [9]. It is important to establish adequate nutritional levels because it can have lifelong effects on offspring. In particular, when maternal protein intake is too low or too high, birth BW is reduced because of placental dysfunction and intrauterine growth retardation, which affects postnatal growth and feed efficiency [5, 53]. In this study, increased CP intake during pregnancy improved the birth BW, ADG, final BW, and FCR of male offspring (Table 6), resulting in a significant increase in carcass weight (Table 7). Beaty et al. [54] reported that the weaning weight of calves improved when the supply of protein increased during pregnancy, and Underwood et al. [3] support our findings by reporting that male offspring born on improved pastures (protein 6% to 11%) have increased weaning BW, final BW and carcass weight compared to offspring born on native pasturelands (protein 5.4% to

6.5%). Carvalho et al. [55] reported that protein supplementation (CP 40%, 3.5 g/kg of body weight) from mid-pregnancy positively affected the energy metabolism and favored the hypertrophic processes of the skeletal muscle in offspring. In addition, there are reports that birth BW and offspring growth are improved by supplementation with protein before delivery [1, 49]. On the other hand, there is evidence that protein restriction during pregnancy negatively affects muscle development of offspring [56, 57, 58]. In other words, the level of protein intake during pregnancy is considered an important factor in growth of offspring. The various amino acids in the protein stimulate the secretion of growth hormone and insulin and regulate protein synthesis in skeletal muscle and placenta by enhancing oxidative and cellular redox capacity [59]. It has also been shown to promote blood vessel angiogenesis and growth, improve blood flow across the placenta, and increase maternal-fetal nutrient transfer [5, 60]. It has been speculated that these effects may eventually positively affect fetal development and postnatal growth. In addition, birth weight is highly related to weaning weight and ADG [61]. Song et al. [62] analyzed the data of 1,907 steers and reported that birth weight was significantly positively correlated with weaning weight, carcass weight, and rib eye area. Therefore, the increase in birth weight due to increased CP intake during pregnancy may have affected final weight and carcass weight. However, in a study by Wilson et al. [45], protein supplementation (128% of protein requirement) for 78 d before delivery did not affect the birth BW and growth of the offspring. Therefore, it was judged that the performance of the fetus might differ depending on the time of protein supplementation, which may be related to the developmental sequence of muscle fibers and adipose tissue according to the growth stage of the fetus [7].

It is well known that stromal vascular cells are a major source of adipogenic cells in skeletal muscle [63, 64], and proteins influence fetal angiogenesis [9, 65]. We hypothesized that increased CP intake during pregnancy could improve proliferation of intramuscular

adipocytes in fetal. However, there was no difference in the degree of intramuscular fat in male offspring with or without increased CP intake in this study, but rather a significant difference in back fat thickness. Similar to our study, offspring supplemented with better protein during pregnancy showed increased back fat thickness and number of subcutaneous fat cells (cells/field) without any difference in intramuscular fat and increased insulin sensitivity [3, 45]. However, in a study by Larson et al. [8], supplementation of protein cubes (0.45 kg/d dry matter, protein 28%) during winter grazing of pregnant cows improved the intramuscular fat content of the offspring. Recent evidence suggests that intramuscular adipocytes behave differently from subcutaneous and visceral adipocytes. [66, 67, 68, 69]. Moreover, intramuscular adipocytes share a common progenitor cell with myogenic cells and are in a competitive relationship [70]. It is presumed that extreme nutritional deficiencies, such as winter grazing, induce the differentiation of fetal intramuscular adipocytes, which may have a greater fetal programming effect, but this is inaccurate. There is evidence that increased CP intake during pregnancy can improve adipogenesis in offspring. However, the mechanisms regulating the differentiation of intramuscular adipocytes have not yet been established, and this should be further discussed and studied.

Economic efficacy

Fetal programming through maternal nutrition can improve offspring performance and provide economic benefits. In this study, although the costs of commercial concentrates for pregnant cows and male offspring increased slightly, increased CP intake during pregnancy increased gross receipts and net income by improving the carcass weight of the offspring. Larson et al. [8] reported that the USDA choice ratio and carcass weight of the offspring increased due to protein supplementation during the pregnancy period, resulting in an increase of \$30/head

revenue; Summers et al. [71] reported that the feedlot purchase price increased because the calf's weight increased with dried distiller's grain with solubles-based protein supplementation. In addition, Stalker et al. [48] reported that maternal protein supplementation increased weaning BW and survival rate of calves, resulting in increased net income. Studies have shown that increased CP intake of maternal can improve the growth and BW of offspring, thereby increasing their economic benefits.

CONCLUSION

Through our study, it is judged that adipogenesis begins in fetal skeletal muscle in at least mid-pregnancy (4 to 6 months). Based on this, a scientific approach is needed to produce high-quality beef through fetal programming effectively. While excessive nutrient over nutrition during pregnancy can negatively affect offspring, 5% increased CP intake during pregnancy can increase profitability through higher meat production yield (carcass weight) by improving the growth of the fetus and offspring. Simultaneously, there is a possibility of increasing subcutaneous fat rather than intramuscular fat; therefore, additional research should be conducted to compensate for these problems.

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651 **Table 1.** Ingredients and chemical composition of pregnant cow experimental diets (As-fed basis)

Item	Concentrate I	Concentrate II	Rice straw
..... Ingredient composition (%).....			
Corn grain	13.12	15.43	
Wheat grain	12.00	15.00	
Cane molasses	3.00	4.00	
Cassava residue	2.00	-	
Wheat flour	6.00	10.00	
Wheat bran	11.70	-	
Corn gluten feed	20.99	23.00	
Soybean	3.65	9.80	
Sunflower seed	-	6.00	
Rapeseed	7.00	6.00	
Corn germ	3.00	-	
Palm kernel	9.00	-	
Corn-DDGS	4.00	6.00	
Salt dehydrated	0.60	0.80	
Limestone	2.20	2.82	
Vit Premix ¹	0.15	-	
Min Premix ²	0.30	-	
Beta-Carotene	0.05	0.05	
Other	1.24	1.10	
Total	100.00	100.00	
..... Chemical composition (%).....			
TDN ³	70.13	72.75	41.51
Dry matter	88.55	88.29	93.65
Crude protein	14.08	18.15	4.08
Ether extract	2.90	2.66	1.85
Crude fiber	6.74	6.61	32.01
Ca	1.17	1.21	0.24
P	0.50	0.49	0.14
Crude ash	6.55	6.77	11.42

DDGS, Dried Distiller's Grains with Solubles.

¹Vitamin premix provided the following quantities of vitamins per kilogram of the diet: vitamin A, 10,000 IU; vitamin D₃, 1,500 IU; vitamin E, 25 IU; ²Mineral premix provided the following quantities of minerals per kilogram of the diet: Fe, 50 mg; Cu, 7mg; Zn, 30 mg; Mn, 24 mg; I, 0.6 mg; Co, 0.15 mg; Se, 0.15 mg; ³TDN: total digestible nutrients (calculated value).

Table 2. Ingredients and chemical composition of male offspring experimental diets (As-fed basis)

Item	Concentrate		Roughage	
	Growing	Fattening	Timothy hay	Rice straw
Corn grain	25.00	31.99		
Wheat grain	8.00	10.00		
Rice	-	3.00		
Cane molasses	3.00	4.00		
Wheat flour	-	1.00		
Wheat bran	6.80	7.00		
Rice bran	-	2.00		
Corn gluten feed	21.30	15.32		
Soybean Meal	5.00	4.00		
Coconut meal	2.95	-		
Palm kernel meal	12.00	12.00		
Corn-DDGS	7.84	-		
Beet pulp	-	-		
Corn cob	-	-		
Lupin flake	3.00	4.00		
Cottonseed	-	2.00		
Protected fat	-	0.06		
Salt dehydrate	0.68	0.70		
Limestone	2.95	2.05		
Sodium bicarbonate	0.30	0.40		
MSG-CMS ¹	0.50	-		
Vitamin premix ²	0.10	0.10		
Mineral premix ³	0.10	0.10		
Feed additives	0.48	0.28		
Total, %	100.00	100.00		
..... Chemical composition (%)				
TDN ⁴	71.46	73.51	56.08	40.10
Dry matter	88.15	87.65	91.25	93.15
Crude protein	16.15	13.29	10.05	4.15
Ether extract	3.85	4.62	1.85	1.92
Crude fiber	6.85	5.95	29.15	31.40
Ca	1.42	0.98	0.27	0.27
P	0.5	0.45	0.27	0.11
Crude ash	7.33	6.52	8.25	13.11

DDGS, Dried Distiller's Grains with Solubles.

¹MSG-CMS: condensed molasses soluble-mono sodium glutamate; ²Vitamin premix provided the following quantities of vitamins per kilogram of the diet: vitamin A, 10,000 IU; vitamin D₃, 1,500 IU; vitamin E, 25 IU; ³Mineral premix provided the following quantities of minerals per kilogram of the diet: Fe, 50 mg; Cu, 7mg; Zn, 30 mg; Mn, 24 mg; I, 0.6 mg; Co, 0.15 mg; Se, 0.15 mg; ⁴TDN: total digestible nutrients (calculated value).

Table 3. Primers for genes analyzed by real-time quantitative polymerase chain reaction

Gene	Gene abbreviation	Accession No.	Forward sequence	Reverse sequence
40S ribosomal protein S9	RPS 9	DT860044	GAGCTGGGTTTGTCTGCAAAA	GGTCGAGGCGGGACTTCT
Myogenin	MyoG	AF091714	AGAAGGTGAATGAAGCCTTCGA	GCAGGCGCTCTATGTACTGGAT
myosin heavy chain 1	MHC 1	AB059400	CCCACCTTCTCCCTGATCCACTAC	TTGAGCGGGTCTTTGTTTTTCT
myosin heavy chain 2A	MHC 2A	AB059398	CCCCGCCCCACATCTT	TCTCCGGTGATCAGGATTGAC
CCAAT/enhancer binding protein β	C/EBP β	NM_176788	CCAGAAGAAGGTGGAGCAACTG	TCGGGCAGCGTCTTGAAC
peroxisome proliferator activated receptor γ	PPAR γ	NM_181024	ATCTGCTGCAAGCCTTGGA	TGGAGCAGCTTGGCAAAGA
stearoyl-CoA desaturase	SCD	AB075020	TGCCCAACCACAAGTTTTCAG	GCCAACCCACGTGAGAGAAG

Table 4. Changes in body weight and length of fetal by gestation period of Hanwoo

Item	Days of gestation			p-value
	90	180	270	
Body weight, kg	0.42±0.04 ^c	4.6±0.84 ^b	19.9±1.43 ^a	0.001
Body length, cm	25.5±0.95 ^c	51.7±2.64 ^b	84.0±1.35 ^a	0.001

^{a,b,c} Means without same superscripts within a row are significantly different ($p < 0.05$).

Table 5. Effects of increased CP intake during pregnancy period on the cow growth performance, subsequent reproduction, and calf birth BW in Hanwoo

Item	CON (n = 34)	TRT (n = 34)	p-value
Months of age	64.96±8.55	61.90±6.03	0.682
Initial BW (30 d of pregnant), kg	545.18±57.91	557.74±47.82	0.333
Final BW (30 d of delivery), kg	556.06±59.49	566.35±47.82	0.434
Average daily gain, kg	0.12±0.15	0.10±0.19	0.541
Dry matter intake, kg/d	8.29±0.00	8.29±0.00	-
Concentrate I	3.54±0.00	2.66±0.00	-
Concentrate II	-	0.88±0.00	-
Rice straw	4.75±0.00	4.75±0.00	-
Calf birth BW			
Female, No	12	17	-
Birth BW	24.89±2.61 ^b	28.61±2.52 ^a	0.001
Male, No	22	17	-
Birth BW	27.45±2.52 ^b	30.65±3.14 ^a	0.002
Reproductive Performance			
No. of SPC ¹	1.43±0.59	1.50±0.77	0.619
Conception rate ^{1st} , %	62.8	63.9	-

SPC, Services per conception.

^{a,b} Means with difference superscripts in the same row are significantly different ($p < 0.05$).

Table 6. Effects of increased CP intake during pregnancy period on the growth performance of male offspring in Hanwoo

Item	CON-O (n = 22)	TRT-O (n = 17)	p-value
Growing (3 - 13 months of age)			
Initial BW, kg	96.32±24.65	110.71±23.79	0.072
Final BW, kg	347.96±50.56 ^b	376.60±33.65 ^a	0.047
Average daily gain, kg	0.80±0.09	0.84±0.12	0.228
Dry matter intake, kg ·d ⁻¹	6.64±0.00	6.64±0.00	-
Concentrate	3.40±0.00	3.40±0.00	-
Timothy	3.24±0.00	3.24±0.00	-
Feed conversion ratio	8.45±1.02	8.04±1.10	0.246
Fattening (14 - 30 months of age)			
Initial BW, kg	347.96±50.56	376.60±33.65	0.047
Final BW, kg	732.10±84.77 ^b	823.20±91.29 ^a	0.003
Average daily gain, kg	0.75±0.21	0.88±0.22	0.083
Dry matter intake, kg ·d ⁻¹	9.04±0.02	9.05±0.02	-
Concentrate	7.56±0.02	7.57±0.02	-
Rice straw	1.48±0.00	1.48±0.00	-
Feed conversion ratio	13.27±5.60	11.10±3.43	0.145
Total (3 - 30 months of age)			
Initial BW, kg	96.32±24.65	110.71±23.79	0.072
Final BW, kg	732.10±84.77 ^b	823.20±91.29 ^a	0.003
Average daily gain, kg	0.77±0.10 ^b	0.86±0.12 ^a	0.019
Dry matter intake, kg ·d ⁻¹	8.09±0.01	8.10±0.01	-
Concentrate	5.92±0.01	5.93±0.01	-
Rice straw	2.17±0.00	2.17±0.00	-
Feed conversion ratio	10.71±1.55 ^a	9.59±1.46 ^b	0.026

^{a,b} Means with difference superscripts in the same row are significantly different ($p < 0.05$).

Table 7. Effects of increased CP intake during pregnancy period on the carcass traits of male offspring in Hanwoo

Item	CON-O (n=22)	TRT-O (n=17)	p-value
Yield traits			
Carcass weight, kg	424.00±52.90 ^b	481.29±57.57 ^a	0.003
Back fat thickness, mm	12.23±5.15	15.65±5.96	0.069
Rib eye area, cm ²	91.45±8.73	95.24±10.50	0.240
Yield index	62.05±1.73 ^a	60.65±1.84 ^b	0.021
Yield grade score ¹	2.14±0.77 ^a	1.59±0.71 ^b	0.028
Quality traits			
Marbling score	5.00±2.12	5.71±1.69	0.254
Meat color	4.55±0.51	4.47±0.51	0.654
Fat color	3.00±0.00	2.94±0.24	0.332
Texture	1.59±0.59	1.65±0.79	0.808
Maturity	2.18±0.39	2.24±0.44	0.695
Quality grade score ²	3.32±1.04	3.82±0.95	0.105
Auction price (USD ³ /kg)	17.46±3.16	18.29±2.28	0.352

¹ Yield grade score: Grade A = 3, grade B = 2, and grade C = 1; ² Quality grade score: Grade 1⁺⁺ = 5, grade 1⁺ = 4, grade 1 = 3, grade 2 = 2, and grade 3 = 1; ³ USD: United States dollar.

^{a,b} Means with difference superscripts in the same row are significantly different (p < 0.05).

Table 8. Effects of increased CP intake during pregnancy period on the economic efficacy in Hanwoo

Item	CON	TRT	p-value
	----- USD ¹ /head -----		
Gross receipts ¹ (A)	7,463.83±2,026.98 ^b	8,801.35±1,779.75 ^a	0.035
Operating costs (B)			
Feed costs ²	3,263.94±4.14 ^b	3,270.52±3.78 ^a	0.001
Commercial concentrate	2,262.35±4.14 ^b	2,268.93±3.78 ^a	0.001
Pregnant cows	271.11±0.00 ^b	274.66±0.00 ^a	0.001
Male offspring	1,991.24±4.14 ^b	1,994.27±3.78 ^a	0.023
Roughage	1,001.59±0.00	1,001.59±0.00	
Pregnant cows	239.40±0.00	239.40±0.00	-
Male offspring	762.19±0.00	762.19±0.00	-
Other ³	632	632	-
Net income (C=A-B)	3,567.89±2,024.31 ^b	4,898.83±1,777.19 ^a	0.034

USD, United States dollar;

¹Selling price of carcass and by product; ²Commercial concentrate: pregnant cows = 0.32 USD/kg, top dressing = 0.34 USD/kg, calf = 0.36 USD/kg, growing = 0.34 USD/kg, early fattening = 0.35 USD/kg, late fattening = 0.36 USD/kg, roughage: timothy = 0.53 USD/kg, rice straw = 0.21 USD/kg; ³Other: water, Power and Fuel cost (32.78 USD), Veterinary and medicine cost (29.30 USD), Automobile cost (40.30 USD), Farm implements cost (167 USD), Farm building and facilities cost (122 USD), Miscellaneous materials cost (73.87 USD), Interest on borrowed capital (60.36 USD), Land rent (4.34 USD), Hired labor cost (44.44 USD), Excretion disposal cost (22.90 USD), Production management cost (18.28 USD).

^{a,b} Means with difference superscripts in the same row are significantly different (p < 0.05).

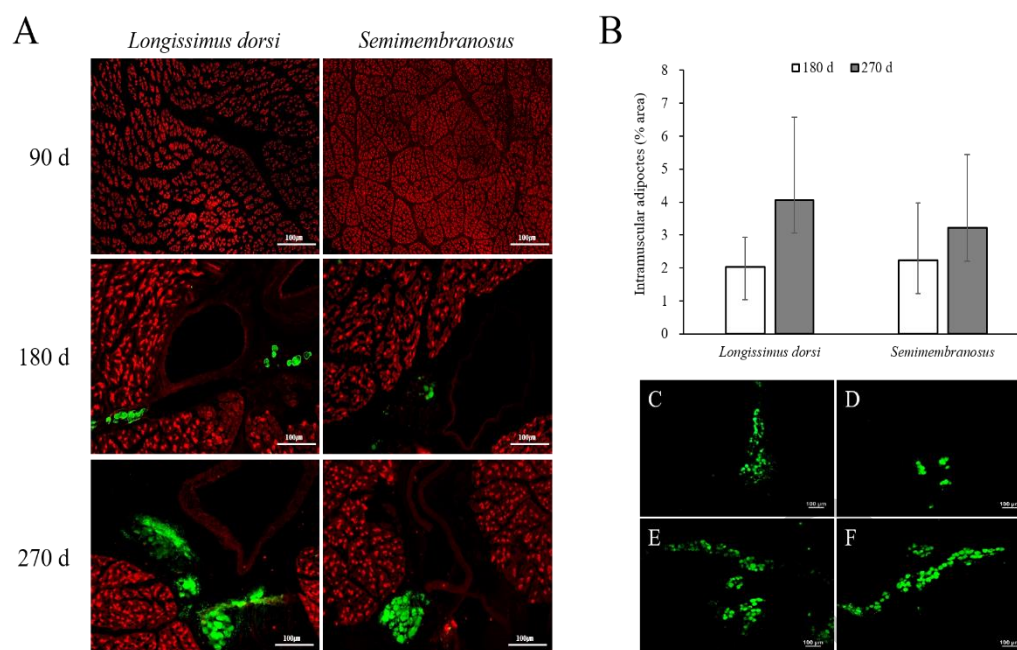


Fig. 1. Immunofluorescence staining (A) and the adipocyte area (B) in the fetal skeletal muscle by pregnancy period of Hanwoo (\pm SD). Representative images of 180 d fetal LD (C), 180 d fetal SM (D), 270 d fetal LD (E), and 270 d fetal SM (F) stained with BODIPY at 100-fold magnification. Green: BODIPY, 4, 4 – difluoro - 1, 3, 5, 7, 8 – pentamethyl – 4 – bora - 3a, 4a – diaza – s - indacene (Lipid); Red: MLC, myosin light chain (muscle).

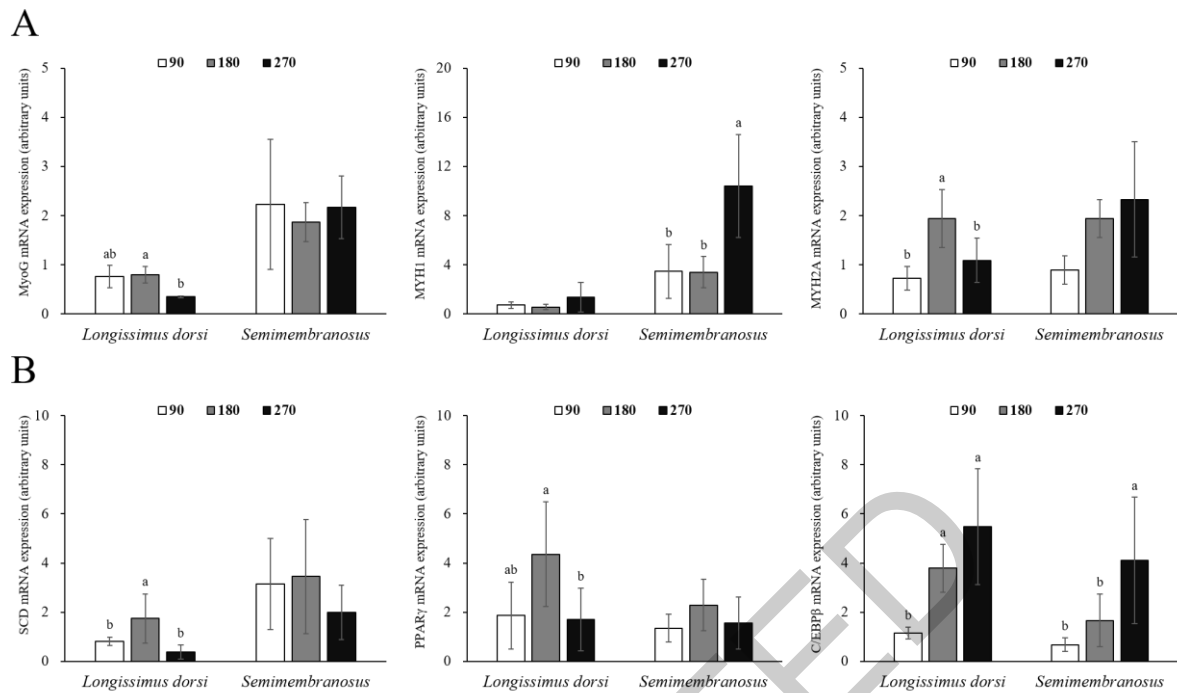


Fig. 2. Expression of genes related to myogenesis (A) and adipogenesis (B) in the fetal skeletal muscle by pregnancy period of Hanwoo

MyoG, myogenin; MYH 1, myosin heavy chain 1; MYH 2A, myosin heavy chain 2A; SCD, stearoyl-CoA desaturase; PPAR γ , peroxisome proliferator activated receptor γ ; C/EBP β , CCAAT/enhancer binding protein β .

^{a,b}Means without same superscripts within a row are significantly different ($p < 0.05$).