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Original Research Article

Vitamin A supplementation downregulates *ADH1C* and *ALDH1A1* mRNA expression in weaned beef calves

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ABSTRACT

Our previous studies demonstrated that oral vitamin A supplementation during late-stage pregnancy and the neonatal stage enhances birth weight, growth performance, and mRNA expression related to muscle and preadipocyte development in beef cattle. The alcohol dehydrogenase 1C (*ADH1C*) *c.-64T > C* genotype also correlated with vitamin A concentration in beef production. This study aimed to investigate the effects of vitamin A supplementation on the muscle development and vitamin A metabolism in weaned beef calves with different *ADH1C* genotypes. Twenty male calves (90 d of age; initial BW: 89.03 kg [SD 8.60]) were stratified according to *ADH1C* genotype and vitamin A treatment (duration: 3 months) and randomly assigned to 4 groups with a 2 × 2 factorial arrangement. Vitamin A treatments included the following: control (10,000 IU/kg of as-fed, a. TT type; b. TC type); treatment (40,000 IU/kg of as-fed, c. TT type; and d. TC type). Parameters including BW, FI, blood, longissimus dorsi muscle, and liver status during the experimental period were analyzed using the generalized linear model (GLM) procedure and Tukey's test by SAS 9.4 program. Serum vitamin A was significantly increased ($P < 0.05$) in the vitamin A treatment group at 4 and 6 months of age. TT type calves showed higher serum vitamin A concentration ($P < 0.05$) than the TC type calves. Serum triglyceride and non-esterified fatty acid (NEFA) levels increased ($P < 0.05$) in the treatment group compared with the control at 6 months of age. However, BW, ADG and FI showed no differences between the groups. In addition, mRNA expression in longissimus dorsi muscle revealed upregulation of paired box 7 (*PAX7*) ($P < 0.05$) after the vitamin A treatment period based on biopsy results. Both *ADH1C* and aldehyde dehydrogenase (*ALDH*) *1A1* mRNA expression was downregulated ($P < 0.01$) by vitamin A supplementation. The TC type of *ADH1C* showed higher mRNA expression than the TT type. However, no effect was observed on adipogenic mRNA expression (pre-adipocyte factor-1 [*PREF-1*], peroxisome proliferator-activated receptor gamma [*PPARγ*], fatty acid binding protein 4 [*FABP4*]) in all groups. Our findings suggest that weaned calves treated with vitamin A may promote the storage of satellite cells by elevating *PAX7* gene expression in the muscle. The TC type calves may show increased capacity for vitamin A metabolism, which can be used in genetically customizing feed management to maximize beef production in the calves.

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1. Introduction

During fetal development, myogenic progenitor cells differentiated from stem cells further differentiate into myocytes and satellite cells (Du et al., 2010). Satellite cells remain quiescent during postpartum muscle development (Kuang et al., 2007). A previous study reported that the myoblasts proliferated and differentiated

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into muscle fibers until 180 d of gestation, and the total number of muscle fibers in the body were determined at this stage (Russell and Oteruelo, 1981). Thereafter, existing muscle fibers continue to hypertrophy (Stickland, 1978), while the proliferation and differentiation of satellite cells into new myocytes are activated by nutritional or physiological stimulation, followed by fusion with muscle fibers to complete the development and maintenance of postnatal muscle tissue (Yin et al., 2013).

Vitamin A is an essential nutrient for cattle, and plays a role in the formation of many body tissues as well as the development of vision and immune function (Bonet et al., 2003). Alcohol dehydrogenase (*ADH*) is the crucial enzyme catalyzing the transformation of retinol (active form of vitamin A) to retinaldehyde, and further oxidation to retinoic acid (RA) by aldehyde dehydrogenase (*ALDH*) (Duester, 2000). In the nucleus, RA binds to the retinoic acid receptors (*RAR*)/retinoid X receptors (*RXR*), and the retinoic acid response element (RARE) which regulates transcription of target genes (Germain et al., 2002; Peng et al., 2021). Wang et al. (2018) reported that vitamin A injection into newborn calves increased the number of satellite cells at 2 months of age. Previous studies in our laboratory revealed that vitamin A supplementation in late pregnancy and during the neonatal stage (birth to 2 months of age) upregulated the expression of myogenic mRNA markers such as myoblast determination protein 1 (*MYOD*) and myogenic factor 6 (*MYF6*) (Jo et al., 2020; Peng et al., 2020a). Hence, the study implied that vitamin A supplementation is strongly associated with muscle development in postnatal calves.

ADH1 is the primary enzyme that oxidizes retinol to retinaldehyde and the mRNA and protein are expressed at high levels in the liver (Molotkov et al., 2002). Among the members of this enzyme family, ADH1C has the highest conversion efficiency to retinol (Höög et al., 2001). Ward et al. (2012) identified a new single nucleotide polymorphism (SNP) within the promoter region of *ADH1C* and found that the TT genotype of *ADH1C c.-64T > C* is associated higher levels of intramuscular fat (IMF) than the CC genotype in Angus crossbred steers when vitamin A is limiting. A subsequent study revealed that the liver expressed different ADH1C protein levels in various *ADH1C* genotypes under vitamin A restriction (Krone et al., 2016). However, when vitamin A was restricted in Korean native steers, the steers carrying the TC genotype expressed a significantly higher level of IMF than in TT genotype (Peng et al., 2017). Although the effects of *ADH1C* genotype on Angus steers and Korean native steers are disputed, the findings implied that vitamin A and *ADH1C* genotype exhibit a specific relationship with beef production. According to the existing studies, vitamin A regulates the IMF deposition and muscle development in beef cattle (Peng et al., 2021). Studies investigating *ADH1C* genotype of cattle are limited to vitamin A restriction and IMF deposition. However, there is paucity of knowledge regarding vitamin A supplementation and muscle development in beef cattle with different *ADH1C* genotypes.

In the current study, we hypothesized that vitamin A supplementation promotes muscle development in weaned beef calves, and the effects vary depending on the *ADH1C* genotype. Therefore, the aim of this study was to investigate the effect of vitamin A supplementation and *ADH1C* genotype on muscle development in weaned Korean native calves.

2. Materials and methods

2.1. Animals and management

All experimental procedures were carried out in accordance with the "Guidelines for Care and Use of Experimental Animals" of Konkuk University (Approval no: KU19095). The animals used in this experiment were selected from a pool of Korean native calves ($n = 50$). According to our previous study, all calves were separated from dams immediately after birth and given commercial colostrum (3.6 L, 225 g in 625 mL of 45 °C water, Headstart, Saskatoon Colostrum Company Ltd., Saskatoon, SK, CA) within 12 h, followed by oral vitamin A supplementation (45,000 IU/d, retinyl acetate, Hanyou Feed, Wuxi, China) until 2 months of age (weaned) (Ahmadi et al., 2021; Peng et al., 2020a). For grouping, we selected the target calves based on BW, *ADH1C* genotype (*ADH1C c.-64T > C*; TT type and TC type) and serum vitamin A concentration at 75 d of age from the pool of the calves. Twenty male calves (age: 75 d; BW: 74.5 kg [SD 7.2]) were then selected and randomly assigned to the control group (TT type: $n = 5$; TC type: $n = 5$) and the vitamin A treatment group (TT type: $n = 5$; TC type: $n = 5$). Each calf was transferred to an independent aluminum alloy pen (240 cm long x 150 cm wide x 180 cm high) and the experiment was started at 90 d of age (initial BW: 89.03 kg [SD 8.60]) after a one-week feed adaptation period (from 83 d of age). The feedstuff used in the experiment comprises 20% forage (oat hay) and 80% concentrate (DH Vital Feed, Gyeonggi-do, Republic of Korea). The diet was formulated according to the nutrient requirements for beef cattle recommended by the Korean Feeding Standard for Hanwoo (Council KFSE, 2017) and the Japanese Feeding Standard for Beef Cattle (NARO, 2008). The composition and nutrient contents of the basal diet are shown in Table 1. CP, EE, and ash contents were measured according to AOAC (2016). NDF and ADF contents were measured by following the methods of Van Soest et al. (1991). Ca and P contents were measured using a Perkin Elmer Avio 200 ICP-OES (Perkin Elmer, Shelton, CT, USA) as described in AOAC (2016). Vitamin A content was measured as described by Peng et al. (2019). The experimental diets were prepared by supplementing different amounts of retinyl acetate (5×10^5 IU/g, Hanyou Feed, Wuxi, China) to the concentrated feed and customized by the feed company. During the adaptation period, all calves were fed a diet consisting of 10,000 IU/kg of vitamin A (as-fed). During the experimental period, the treatment group was fed with a diet containing a higher concentration of vitamin A (40,000 IU/kg vitamin A, as-fed), and the control group was maintained at 10,000 IU/kg vitamin A (as-fed). Besides, each calf was supplied with separate and clean buckets for feed and water (ad libitum). Also, the dry sawdust in the pen was replaced once a week during the experimental period. The health status of the calves was checked every day during the experimental period, those who involved diarrhea and respiratory disease were treated by the related administration under the guidance of a veterinarian. All calves were vaccinated with a commercial inactivated viral vaccine (CattleMaster 4, Zoetis, Sandton, ZA) and treated with an antiparasitic drug (Ivomec-F, Boehringer Ingelheim, Duluth, GA, USA). The remains of feed from the previous day were collected and weighed daily before morning feeding to calculate FI. The BW was measured before the morning feeding every month, and the ADG and feed efficiency (gain:feed of DM) were calculated over a vitamin A treatment period of 3 to 6 months of age.

2.2. Preparation and analysis of blood samples

Blood samples were collected from the jugular vein of each calf at 08:00 before feeding once a month. The samples were transferred to 2 tubes to collect serum and whole blood separately. The tubes included serum tubes with clot activator (BD Vacutainer, Franklin Lakes, NJ, USA) wrapped in a tin foil, and another tube containing 7.2 mg/kg of ethylenediaminetetraacetic acid (EDTA, BD Vacutainer, Franklin Lakes, NJ, USA) for whole blood samples. All tubes were placed on ice and transported to the laboratory. The blood samples in the serum tubes were further coagulated for 1 h at room temperature and separated by centrifugation at 2,700 × *g* for

Table 1
Composition and nutrient content of the basal diet (%, DM basis).

Item	Contents
Ingredients	
Oat hay	17.65
Wheat bran	19.10
Corn	16.49
Soybean meal	10.54
Alfalfa	9.91
Wheat	7.08
Soybean bran	3.54
Yeast culture[1]	1.06
Soybean oil	0.99
Calcium carbonate	0.28
Salt	0.21
Sodium bicarbonate	0.07
Magnesium oxide	1.42
Vitamin-mineral premix[2]	0.07
Nutrient contents	
CP	13.20
EE	2.76
Ash	5.82
NDF	26.02
ADF	13.51
Ca	0.82
P	0.41
Vitamin A[3], IU/kg	553.80

[1] Commercial fermentation product (Original XP, Diamond V, Cedar Rapids, IA, USA).
[2] Premix provided the following for per kilogram of diet: vitamin A, 1,660 IU; vitamin D_3, 200 IU; vitamin E, 250 μg; vitamin B_1, 410 μg; vitamin B_2, 400 μg; vitamin B_{12}, 5 μg; niacin, 500 mg; folic acid, 450 mg; methionine, 5 mg; manganese, 1.55 mg; iron, 1.5 mg; copper, 1.7 mg; zinc, 2.1 mg; selenium, 215 μg.
[3] Original concentration of vitamin A in feedstuff, excluding the vitamin A in premix.

15 min at 4 °C. The obtained serum was divided into 1.5-mL brown microtubes and stored in a deep freezer (−80 °C) for future analysis. The whole blood samples in EDTA-containing tubes were stored in the refrigerator (4 °C) for DNA extraction.

2.3. DNA extraction

The method used to extract DNA in this study was described previously (Peng et al., 2017) with minor modifications. Specifically, DNA from calves was extracted from whole blood samples within 24 h of blood collection. The fresh blood sample (200 μL) and 1.2 mL of red blood cell lysis buffer (Sigma-Aldrich, Seoul, Republic of Korea) were added into a 1.5 mL microtube. After 10 s of vortexing, the sample was left for 5 min at room temperature for red blood cell lysis. The sample was then centrifuged at 2,000 × *g* for 5 min at 20 °C and the supernatant was discarded. Next, a 300 μL lysis buffer (mixed with 10 mmol/L Tris-Cl pH 8.0, 10 mmol/L EDTA pH 8.0, 100 mmol/L NaCl) and 2% sodium dodecyl sulfate (SDS) in distilled water were added and briefly vortexed to release DNA. Then, 5 μL proteinase K (20 mg/mL, iNtRON Biotechnology, Seongnam, Republic of Korea) was added to the sample, and after brief vortexing, the sample was incubated for 3 h at 56 °C, followed by the addition of 300 μL ammonium acetate solution (7 mol/L, Biosesang, Seongnam, Republic of Korea) to the tubes and centrifugation at 16,000 × *g* for 15 min at 20 °C to precipitate the protein. The supernatant was transferred to the new 1.5 mL microtube, and an equal volume of cold isopropanol was added and vortexed briefly. The mixture was incubated for 1 h at −20 °C and centrifuged at 20,000 × *g* for 15 min at 4 °C to precipitate the DNA. After discarding the supernatant, 500 μL of 70% cold ethanol was added to the pellet to wash the DNA extract, followed by centrifugation of the sample at 20,000 × *g* for 5 min at 4 °C. Subsequently, the supernatant was removed, and the obtained pellet was left to dry at room temperature for 2 h. Finally, the sample was dissolved in 50 μL Tris-EDTA buffer and refrigerated at 4 °C, and used within a week.

2.4. ADH1C genotyping

The genotyping of calves was performed in the *ADH1C c.-64T > C* region using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), which was previously described by Peng et al. (2017). The primers used for PCR amplification of the *ADH1C* gene are listed in Table 2. The PCR reaction mixture was prepared in a total volume of 30 μL containing 15 μL of 2 × Taq PCR premix kit (Solgent Co. Ltd, Daejeon, Republic of Korea), 0.5 μL each of forward and reverse primers (10 μmol/L), 2 μL of DNA template and 12 μL of triple-distilled water. The PCR reaction was carried out in a T100 96-well Thermal Cycler (Bio-Rad, Seoul, Republic of Korea) using a program for pre-incubation of samples for 5 min at 95 °C, followed by 35 cycles of amplification, each consisting of 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C, and a final 5 min at 72 °C. Subsequently, 1 μL CutSmart Buffer (New England Biolabs, Hitchin, UK) and 0.2 μL BslI (New England Biolabs, Hitchin, UK) were mixed with 10 μL PCR product and digested for 5 h at 55 °C, in which the C allele was cut into 160 and 93-bp fragments (Ward et al., 2012). The digested fragments were resolved via 2% agarose gel electrophoresis containing Ecodye Nucleic Acid Staining Solution (Biofact, Daejeon, Republic of Korea) at 70 V for 50 min by MupidexU system (Takara Bio, Shiga, Japan) with 100 bp Plus DNA Ladder (Biofact, Daejeon, Republic of Korea), and then visualized under UV light (Fig. 1).

2.5. Serum vitamin A analysis

Serum vitamin A was analyzed as described previously (Peng et al., 2019). Specifically, the analysis was carried out in a dark room with a red light to prevent the oxidation of vitamin A in the sample. Methanol and hexane used in this study were HPLC grade. Ethanol and other chemicals used were analytical grade. Retinyl acetate (>99%, Sigma-Aldrich, Seoul, Republic of Korea) was used as an internal standard. Both ethanol and hexane, and internal standard solution were stabilized with 0.04% butylated hydroxytoluene (BHT, Sigma-Aldrich, Seoul, Republic of Korea). The standard curve (external standard) used in this analysis was constructed by diluting retinol (>99%, Sigma-Aldrich, Seoul, Republic of Korea) to different concentrations (12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10 and 0.05 mg/mL), followed by treatment with an internal standard up to 1 mg/mL each.

Serum samples were thawed at room temperature, followed by the addition of 200 μL of serum to a 2 mL microtube containing 20 μL of internal standard solution, 200 μL of distilled water, and 400 μL of ethanol, and then vortexed vigorously. The samples were extracted with 800 μL of hexane, and vortexed again, followed by centrifugation at 3,500 × *g* for 10 min at 4 °C. The supernatant was transferred to a 1.5-mL brown microtube and evaporated under nitrogen gas, followed by reconstitution with 500 μL 95% methanol. Besides, 20 μL of internal standard solution and 480 μL of ethanol were added to 1.5 mL brown microtube in triplicate. All samples were filtered into the 2 mL screw top vials (Agilent Technologies, Richardson, USA) with a syringe filter (PES, 0.33 mm, 0.22 μm, Millex, Darmstadt, Germany), followed by HPLC (Agilent 1100 series, Waldbronn, Germany). For separation of vitamin A, a stainless-steel Nova-Pak C18 column (4-μm reversed-phase column, 150 mm × 3.9 mm ID, Waters, Dublin, Ireland) was used and set at 20 °C. The mobile phase was 95% methanol at a flow rate of 1 mL/

Table 2
Primer sequences designed for PCR-restriction fragment length polymorphism (PCR-RFLP) and quantitative real-time PCR (qPCR).

Target gene	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Accession number	Annealing temperature, °C
ADH1C (PCR-RFLP)	CAGGGCTTAAAGATCCCAGA	TAGCCAATGCTTGTCTCTCG	NC007304.4	60.0
PAX7	GACAAAGGGAACCGTCTGGA	GGGTAGTGGGTCCTCTCGAA	XM_015460690.2	62.5
MYF5	CGGATCACCAACTCAGAGCA	AGGTTGAGAATCGGTGCTGG	NM_174116.1	62.5
MYOD	AACTGTTCCGACGGCATGAT	GCTGTAGTAAGTGCGGTCGT	NM_001040478.2	63.9
MYOG	CAGGGCTCTTCTAAGCCAGG	CAGAACTGCCCTCTTGCTCT	NM_001111325.1	64.6
MYF6	GAAGGAGGGACAAGCATTGA	GAGGAAATGCTGTCCACGAT	NM_181811.2	60.7
PREF-1	CCTCTTGCTCCTGCTGGCTTTC	AAGGTCACGCACTGGTCACAC	NM_174037.2	58.8
ZFP423	AGGGCTGGTCCAAGGTGGTGTA	TGTGGCTCTGCTGCGGCTTATC	NM_001,101,893	63.9
PPARγ	ACTTTGGGATCAGCTCCGTG	TCCTCATAGTGCGGAGTGGA	NM_181024.2	60.8
FABP4	CGTGGGCTTTGCTACCAG	TGGTTGATTTTCCATCCCAG	NM_174314.2	60.0
ADH1C (qPCR)	GCACCTTCACCCATACACA	AATCCGCAGCCAATGAGACA	NM_001206387.1	63.9
ALDH1A1	CCGTGTGGGTGAACTGCTAT	CCCAGTTCTCGACCATTTCCA	NM_174239.2	65.0
GAPDH	GAAGGTCGGAGTGAACGGAT	GATGGCGACGATGTCCACTT	NM_001034034.2	60.8
RPLP0	GGCAGCATCTACAACCCTGA	CAGATGCGACGGTTGGGTAA	NM_001012682.1	60.8
RPS9	TTCCAGAGCGTTGGCTTAGG	CGGGGTCACATAGGTTTTGC	NM_001206387.1	63.9

ADH1C = alcohol dehydrogenase 1C; *PAX7* = paired box 7; *MYF5* = myogenic factor 5; *MYOD* = myoblast determination protein 1; *MYOG* = myogenin; *MYF6* = myogenic factor 6; *PREF-1* = preadipocyte factor-1; *ZFP423* = zinc finger protein 423; *PPARγ* = peroxisome proliferator-activated receptor gamma; *FABP4* = fatty acid binding protein 4; *ALDH1A1* = aldehyde dehydrogenase 1 family member A1; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *RPLP0* = ribosomal protein lateral stalk subunit P0; *RPS9* = ribosomal protein S9.

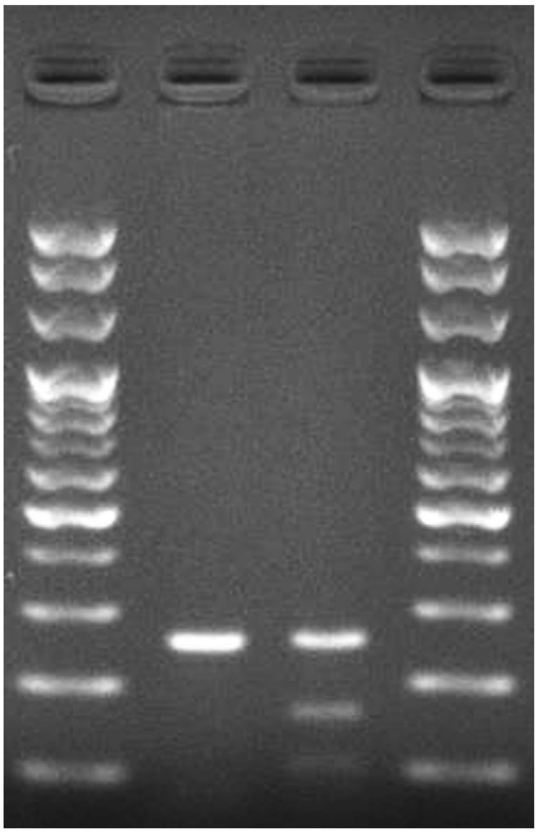

Fig. 1. Agarose gel electrophoresis of *ADH1C c.-64T >C* polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *ADH1C* = alcohol dehydrogenase 1C. Lane 1: 100 bp DNA Ladder; Lane 2: TT type; Lane 3: TC type, Lane 4: 100 bp DNA Ladder.

min. Vitamin A was quantified by measuring the absorption at 325 nm using a DAD-UV lamp. Areas of retinol and retinyl acetate from samples were obtained using the HPLC computer program. The concentration of vitamin A was calculated as the ratio of the individual sample area to the external standard. The result was corrected using the recovery rate of the internal standard. The average recovery was more than 80%. All the serum vitamin A samples were analyzed in duplicate.

2.6. Metabolic profile test (MPT)

The MPT analyses included 11 items. Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (TCHO), albumin (ALB), calcium (CA), magnesium (MG) and triglyceride (TG) were measured with an automated clinical chemistry analyzer (FUJI DRI-CHEM 7000i; Fujifilm, Tokyo, Japan), and the non-esterified fatty acid (NEFA) was measured using an automated analyzer (HITACHI 7600; HITACHI, Tokyo, Japan).

2.7. Muscle tissue and liver biopsy

The longissimus dorsi muscle sample was collected from each calf by biopsy at 6 months of age. The sampling site was located between the 12th and 13th ribs. All the biopsy procedures were carried out by the veterinarian in strict accordance with the "Guidelines for the Care and Use of Experimental Animals" of the Konkuk University (Approval no: KU19095). First, a 5 cm × 5 cm area of the skin was shaved, followed by subcutaneous injection of 2.5 mL 2% lidocaine separately (Jeil Pharmaceutical, Yongin, Republic of Korea) at 4 equidistant points around the surgical site to induce local anesthesia. About 2 g of muscle sample was collected with blunt-end scissors. The muscle sample was then transferred into 5 mL diethyl pyrocarbonate (DEPC)-treated microtubes and immediately placed in liquid nitrogen for rapid freezing. The liver biopsy was performed immediately after the muscle biopsy and was collected through the 10th intercostal space on the right side. After preoperative preparation (as described above), approximately 0.2 g of liver tissue was obtained with Achieve coaxial biopsy systems (14 G, 15 cm, CareFusion, San Diego, USA), and transferred into 2 mL DEPC-treated microtubes and immediately frozen under liquid nitrogen. Thereafter, the calves were managed for a week as recommended by the veterinarian to prevent health issues. The quick-frozen muscle and liver samples in the liquid nitrogen were then transferred to a box full of dry ice before transporting to the laboratory, and then stored in a deep freezer (−80 °C) until analyses.

2.8. Total RNA extraction and cDNA synthesis

The total RNA in muscle and liver tissue was extracted by TRIzol method as described by Peng et al. (2020a). Concretely, all tubes and tips used for RNA extraction and cDNA synthesis were

processed with DEPC-treated water, and other materials were pretreated by RNaseZAP (Sigma-Aldrich, Seoul, Republic of Korea). The collected muscle tissue was ground to a fine powder in liquid nitrogen using pestle and mortar, then the obtained powder was loaded into a 2-mL microtube (each tube contained 0.1 g). Next, 1 mL of TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) was added to microtubes containing muscle and liver samples before homogenization with a T10 basic ULTRA-TURRAX (S10 – 5 G, IKA, Staufen, Germany). The microtubes were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the upper aqueous phase was then transferred into a 1.5 mL microtube, followed by the addition of 200 μL chloroform to each microtube, and then vigorous vortexed. The sample was incubated for 2 min at room temperature before centrifuging at 12,000 × *g* for 15 min at 4 °C. The upper aqueous phase containing RNA was transferred into a 1.5 mL microtube, followed by the addition of 500 μL isopropanol, and vortexed briefly. The solution was incubated for 10 min at room temperature, and the microtubes were centrifuged at 12,000 × *g* for 15 min at 4 °C to precipitate the RNA. Thereafter, the supernatant was removed, and 1 mL 75% ethanol was added to the pellet, followed by vigorous vortexing for washing. The foregoing washing method was repeated once with 100% ethanol, and the supernatant was completely discarded. After drying under vacuum for 15 min, the extracted RNA was redissolved in 30 μL of DEPC-treated distilled water and incubated for 10 min at 60 °C. The RNA purity and concentration were measured by spectrophotometry (NanoDrop 1000, Thermo Scientific, Seoul, Republic of Korea). The RNA integrity was evaluated using the RNA Nano 6000 assay kit (Agilent Technologies, Richardson, USA) on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Richardson, USA). Samples with the RNA integrity number (RIN) ≥ 6.5 were subjected to cDNA synthesis and subsequent quantitative real-time PCR (qPCR) (Fleige and Pfaffl, 2006; Schroeder et al., 2006). The RIN of samples used in this study was 8.1 ± 0.4 (muscle) and 8.3 ± 0.5 (liver).

The cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, Seoul, Republic of Korea). Reverse transcription was performed in a total volume of 20 μL containing 1 μg total RNA, and 4 μL 5 × iScript reaction mix, 1 μL iScript reverse transcriptase, and nuclease-free water. The reverse transcription polymerase chain reaction (RT-PCR) reaction was performed using a T100 96-well Thermal Cycler (Bio-Rad, Seoul, Republic of Korea) under the following cycle conditions: priming for 5 min at 25 °C, reverse transcription for 30 min at 42 °C, and inactivation for 5 min at 85 °C. Following the synthesis, the cDNA was diluted to 200 ng/μL with DEPC-treated water and then stored at −20 °C until qPCR was performed.

2.9. Quantitative real-time PCR

The procedure of the qPCR analysis in this study was conducted based on Taylor et al. (2019) to maximize the data quality. Specific primers for qPCR were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) as shown in Table 2. The reaction mixture used for qPCR was prepared in a total volume of 20 μL containing 10 μL SYBR green qPCR MasterMix (AccuPower 2 × GreenStar qPCR MasterMix, Bioneer, Seoul, Republic of Korea), 0.6 μL of each of forward and reverse primers (10 μmol/L), 2.5 μL of cDNA and 6.3 μL of DEPC-treated water. The qPCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Seoul, Republic of Korea) with the following cycle conditions: initial denaturation for 3 min at 95 °C, followed by 39 amplification cycles, 10 s each at 95 °C, 30 s at applicable temperature for each primer of gene, 30 s at 72 °C, followed by plate reading. After 10 s at 95 °C, melting was performed at 65 and 95 °C with increments of 0.5 °C for 5 s, followed by final plate reading. The amplification efficiencies of all genes were calculated from the standard curve at 10-fold serial dilution and were above 95%. The relative expression of the targeted genes was normalized against the expression of reference genes (*GAPDH* and *RPLP0* for muscle, *GAPDH* and *RPS9* for liver) (Janovick-Guretzky et al., 2007; Wang et al., 2005), and data analysis was carried out in CFX Maestro 1.0 software (Bio-Rad, Seoul, Republic of Korea) using the ΔΔCq method.

2.10. Statistical analysis

All the statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data are expressed as means ± SE. Data generated for growth performance, serum vitamin A concentration, MPT, and mRNA expression were analyzed via 2 × 2 factorial arrangement design. The effects of vitamin A supplementation, *ADH1C* genotype, and their interaction effect on BW, ADG, FI, feed efficiency, serum vitamin A concentration, MPT, and mRNA expression were determined via GLM procedure, followed by mean comparison with Tukey's test. The expression of *ADH1C*, *ALDH1A1*, paired box 7 (*PAX7*) and *MYF6* mRNA expression was assessed using independent (unpaired) sample *t*-tests for unequal variances and GLM procedure with Tukey's test. Significant difference was set at $P < 0.05$, and the tendency was declared at $0.05 \leq P < 0.1$.

3. Results

3.1. Animal growth performance with or without vitamin A supplementation

There were no differences ($P > 0.05$) in BW, ADG, FI and feed efficiency from 3 to 6 months between the control and vitamin A groups (Table 3). There were no differences between TT and TC types of *ADH1C* or in the interaction between vitamin A supplementation and *ADH1C* genotype ($P > 0.05$). In the vitamin A treatment group, no significant difference existed between the 2 genotypes for BW ($P > 0.05$).

3.2. Serum vitamin A concentration and metabolic profile test (MPT) level

After vitamin A supplementation, the serum retinol concentration of calves was greater at 4 and 6 months of age ($P < 0.05$), and tended to increase at 5 months of age ($P = 0.053$) (Table 4). In addition, the retinol concentration of TC type calves was significantly greater than in TT type at 4, 5 and 6 months of age ($P < 0.05$). However, no significant interaction was found between vitamin A treatment and *ADH1C* genotypes in terms of serum vitamin A concentration.

Serum TG and NEFA concentrations of calves supplemented with vitamin A were significantly reduced ($P < 0.05$) at 6 months of age, and the serum AST concentration of TC type calves was significantly lower than in TT type calves ($P = 0.043$) (Table 5). In addition, no significant differences ($P > 0.05$) were observed in other biochemical parameters including ALT, BUN, CRE, GLU, TCHO, ALB, CA and MG.

3.3. Expression of target mRNA in longissimus dorsi muscle and liver

Compared with the control group, the expression of *ADH1C* and *ALDH1A1* mRNA was significantly decreased ($P < 0.01$) in the liver of calves in the vitamin A treatment group (Fig. 2A). The expression of *ADH1C* mRNA in the liver of TC type calves was significantly greater ($P < 0.01$) than in TT type calves, but there was no genotype effect

Table 3
Growth performance parameters in weaned calves from 3 to 6 months of age in control and treatment groups carrying 2 different genotypes.

Item	Control		Vitamin A treatment[1]		SE	*P*-value[2]		
	TT type	TC type	TT type	TC type		Vitamin A (V)	Genotype (G)	V × G
BW, kg								
3 months of age	88.5	89.9	88.4	89.4	5.48	0.944	0.774	0.966
4 months of age	123.6	124.0	121.4	123.6	5.41	0.763	0.774	0.831
5 months of age	161.3	160.6	158.7	160.9	4.31	0.761	0.861	0.737
6 months of age	198.6	198.6	195.1	203.4	6.59	0.892	0.391	0.391
ADG, kg/d								
3 to 4 months of age	1.2	1.1	1.1	1.1	0.07	0.563	0.995	0.525
4 to 5 months of age	1.2	1.2	1.2	1.2	0.09	0.934	0.812	0.804
5 to 6 months of age	1.2	1.3	1.2	1.4	0.12	0.575	0.286	0.390
3 to 6 months of age	1.2	1.2	1.2	1.3	0.07	0.819	0.484	0.306
Feed intake, kg/d of DM								
3 to 4 months of age	3.4	3.5	3.5	3.4	0.05	0.767	0.554	0.391
4 to 5 months of age	5.0	4.9	4.9	4.9	0.12	0.807	0.555	0.637
5 to 6 months of age	5.3	5.4	5.3	5.4	0.11	0.998	0.276	0.942
3 to 6 months of age	4.6	4.5	4.6	4.6	0.06	0.742	0.674	0.957
Feed efficiency, g gain/kg of DM								
3 to 6 months of age	264.0	262.3	255.1	273.8	0.07	0.900	0.426	0.343

[1] Vitamin A treatment (40,000 IU/kg from 3 to 6 months of age, as-fed).
[2] *P*-values for differences between means are shown; significance was declared when $P < 0.05$, and a trend when $0.05 \leq P < 0.1$.

Table 4
Changes in serum vitamin A levels of weaned calves from 3 to 6 months of age in the control and treatment groups carrying 2 different genotypes.

Item	Control		Vitamin A treatment[1]		SE	*P*-value[2]		
	TT type	TC type	TT type	TC type		Vitamin A (V)	Genotype (G)	V × G
Vitamin A concentration (retinol), IU/dL								
3 months of age	103.4	102.9	121.4	100.8	12.68	0.346	0.216	0.239
4 months of age	110.6	103.1	140.5	109.6	7.75	0.017	0.013	0.105
5 months of age	119.2	108.9	142.3	112.8	8.02	0.053	0.007	0.161
6 months of age	116.5	103.6	138.0	116.3	9.15	0.036	0.034	0.560

[1] Vitamin A treatment (40,000 IU/kg from 3 to 6 months of age, as-fed).
[2] *P*-values for differences between means are shown; significance was declared when $P < 0.05$, and a trend when $0.05 \leq P < 0.1$.

($P > 0.05$) on *ALDH1A1* mRNA expression (Fig. 2B). No interaction was found between *ADH1C* genotype and vitamin A treatment ($P > 0.05$). Also, vitamin A supplementation increased ($P < 0.01$) the expression of *PAX7* mRNA in longissimus dorsi muscle of calves more than in the control group (Fig. 3A). The *ADH1C* genotype did not affect ($P > 0.05$) mRNA expression of *PAX7* and *MYF6* (Fig. 3B). In addition, when comparing vitamin A treatment and *ADH1C* genotype, the expression of *MYF6* mRNA in TC type calves was significantly higher ($P < 0.05$) than in TT type calves (Fig. 3C). However, no significant difference ($P > 0.05$) was observed in the expression of other mRNA related to myogenesis and adipogenesis (Appendix Table 1). No significant interaction ($P > 0.05$) existed between vitamin A treatment and *ADH1C* genotypes in case of all mRNA expression related to myogenesis and adipogenesis.

4. Discussion

In the early growth phase of calves after weaning, the BW intuitively reflects the development of muscle, which is usually considered as one of the most important indicators of growth performance. Hence, in order to maximize the productivity of beef cattle, the focus of investigation should be on muscle development. From late gestation until the postnatal growth period, muscle increases in mass predominantly via hypertrophy of the myofibrils (Greenwood et al., 1999, 2000). Retinoic acid was reported to influence multiple physiological functions as well as cell proliferation, differentiation and gene expression in animals (Harrison, 2005; Peng et al., 2021; Ross and Gardner, 1994). In the zebrafish model, RA enhanced muscle differentiation by activating muscle fibroblast growth factor 8 (Fgf 8) (Hamade et al., 2006). Also, studies in rats revealed that vitamin A deficiency decreased the expression of muscle development-related proteins such as MYF5, myogenin (MYOG), and myosin heavy chain (MyHC) (Downie et al., 2005). In addition, supplementation with a combination of vitamins A and E increased the BW and ADG in Holstein steers (Salinas-Chavira et al., 2014). Similarly, previous studies in our laboratory showed that high levels of vitamin A supplementation in late pregnancy cattle and neonatal calf significantly increased the birth weight of offspring and BW by 45 d after birth (Jo et al., 2020; Peng et al., 2020a). Therefore, vitamin A supplementation is highly correlated with muscle development in beef cattle.

Our previous study showed an increase in BW via vitamin A supplementation in weaning calves at 45 d of birth (Peng et al., 2020a). Accordingly, in the current study, we supplemented all calves with extra-high levels of vitamin A from birth until 2 months of age (weaned). Based on previous studies (Jo et al., 2020; Peng et al., 2020a), we supplied the weaned calves with a high level of vitamin A in the treatment group (40,000 IU/kg, as-fed) compared with the basal level in the control group (10,000 IU/kg of vitamin A, as-fed) from 3 to 6 months of age, which was approximately 10-fold the recommended amount (NRC, 2001) in calves. However, we found no significant differences in BW or FI of calves throughout the experiment, probably due to the reduced effect of vitamin A after weaning in the calves (Anderson et al., 1987; Rode et al., 1990). Besides, the supplementation of high levels of vitamin A before weaning may have elevated the vitamin A reserves in calves, resulting in no significant differences in growth performance after weaning. Also, vitamin A concentration in the control group (10,000 IU/kg, as-fed; 2.5-fold higher than the NRC

Table 5
Results of metabolic profile test (MPT) in weaned calves from 3 to 6 months in control and treatment groups carrying 2 different genotypes.

Item	Control		Vitamin A treatment[1]		SE	*P*-value[2]		
	TT type	TC type	TT type	TC type		Vitamin A (V)	Genotype (G)	V × G
AST, U/L								
Initial	117.2	90.2	126.6	91.0	20.76	0.753	0.067	0.790
6 months of age	63.0	56.2	61.6	48.8	5.50	0.338	0.043	0.510
ALT, U/L								
Initial	18.8	17.6	20.6	17.6	1.36	0.424	0.074	0.424
6 months of age	20.8	17.8	20.6	19.0	2.01	0.702	0.096	0.597
BUN, mg/dL								
Initial	8.5	10.0	9.4	12.2	1.77	0.220	0.103	0.602
6 months of age	9.7	10.7	12.9	11.0	1.96	0.162	0.731	0.261
CRE, mg/dL								
Initial	0.7	0.6	0.6	0.8	0.06	0.393	0.256	0.025
6 months of age	0.7	0.6	0.7	0.7	0.04	0.283	0.175	0.158
GLU, mg/dL								
Initial	70.0	80.2	77.6	72.6	6.15	1.000	0.586	0.124
6 months of age	98.4	96.6	91.2	97.0	5.21	0.369	0.579	0.296
TCHO, mg/dL								
Initial	72.8	73.8	73.6	61.8	10.18	0.516	0.531	0.459
6 months of age	113.8	102.0	104.2	93.0	14.72	0.425	0.326	0.979
ALB, g/dL								
Initial	3.1	3.1	3.1	3.0	0.10	0.893	0.504	0.353
6 months of age	3.4	3.3	3.4	3.2	0.10	0.461	0.061	0.621
CA, mg/dL								
Initial	10.7	10.9	10.5	10.3	0.26	0.088	0.925	0.354
6 months of age	10.8	10.6	10.7	10.6	0.22	0.656	0.322	0.823
MG, mg/dL								
Initial	2.4	2.6	2.6	2.5	0.17	0.823	0.709	0.416
6 months of age	2.4	2.5	2.6	2.4	0.15	0.829	0.520	0.144
TG, mg/dL								
Initial	11.2	16.0	15.8	18.8	5.15	0.274	0.250	0.787
6 months of age	16.4	13.0	9.0	11.0	2.45	0.013	0.682	0.127
NEFA, mmol/L								
Initial	161.6	130.4	120.6	138.6	29.92	0.444	0.756	0.257
6 months of age	181.2	183.0	159.8	125.4	17.43	0.009	0.237	0.191

AST = aspartate transaminase; ALT = alanine transaminase; BUN = blood urea nitrogen; CRE = creatinine; GLU = glucose; TCHO = total cholesterol; ALB = albumin; CA = calcium; MG = magnesium; TG = triglycerides; NEFA = non-esterified fatty acid.
[1] Vitamin A treatment (40,000 IU/kg from 3 to 6 months of age, as-fed).
[2] *P*-values for differences between means are shown; significance was declared when $P < 0.05$, and a trend when $0.05 \leq P < 0.1$.

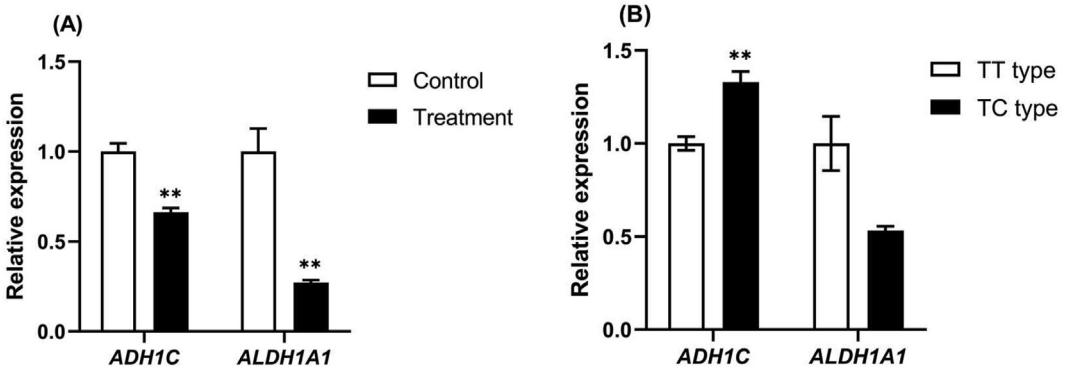

Fig. 2. Expression of target mRNA of liver in weaned calves after vitamin A supplementation. (A) *ADH1C* and *ALDH1A1* mRNA expression in control and vitamin A treatment group. (B) *ADH1C* and *ALDH1A1* mRNA expression in TT and TC type of *ADH1C* genotype. *ADH1C* = alcohol dehydrogenase 1C; *ALDH1A1* = aldehyde dehydrogenase 1 family member A1. Vitamin A supplementation (40,000 IU/kg) in as-fed for 3 to 6 months after weaned. The quantitative real-time PCR values are expressed as fold changes after normalization against the control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S9 (*RPS9*) mRNA. Data are expressed as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. *ADH1C* × treatment × genotype: $P > 0.05$, *ALDH1A1* × treatment × genotype: $P > 0.05$.

recommendation) was adequate to maintain normal growth. Consistent with the present results, Harris et al. (2018) reported that injecting 150,000 and 300,000 IU of vitamin A to Angus calves at birth did not significantly alter growth performance. Thus, our results suggest that compared with commercial feed levels of vitamin A (10,000 IU/kg, as-fed), feeding calves with a diet containing 40,000 IU/kg vitamin A from 3 to 6 months of age did not cause any significant changes in BW, FI and ADG.

Our previous study showed that a high level of oral vitamin A tended to increase the serum vitamin A concentration in Korean native calves (Peng et al., 2020a). Conversely, another study in Angus noted that injecting 150,000 or 300,000 IU of vitamin A to neonatal calves did not change serum retinol concentrations compared with the control group. However, injection of 300,000 IU of vitamin A resulted in lower levels of serum vitamin A than in the group treated with 150,000 IU (Harris et al., 2018). In the current

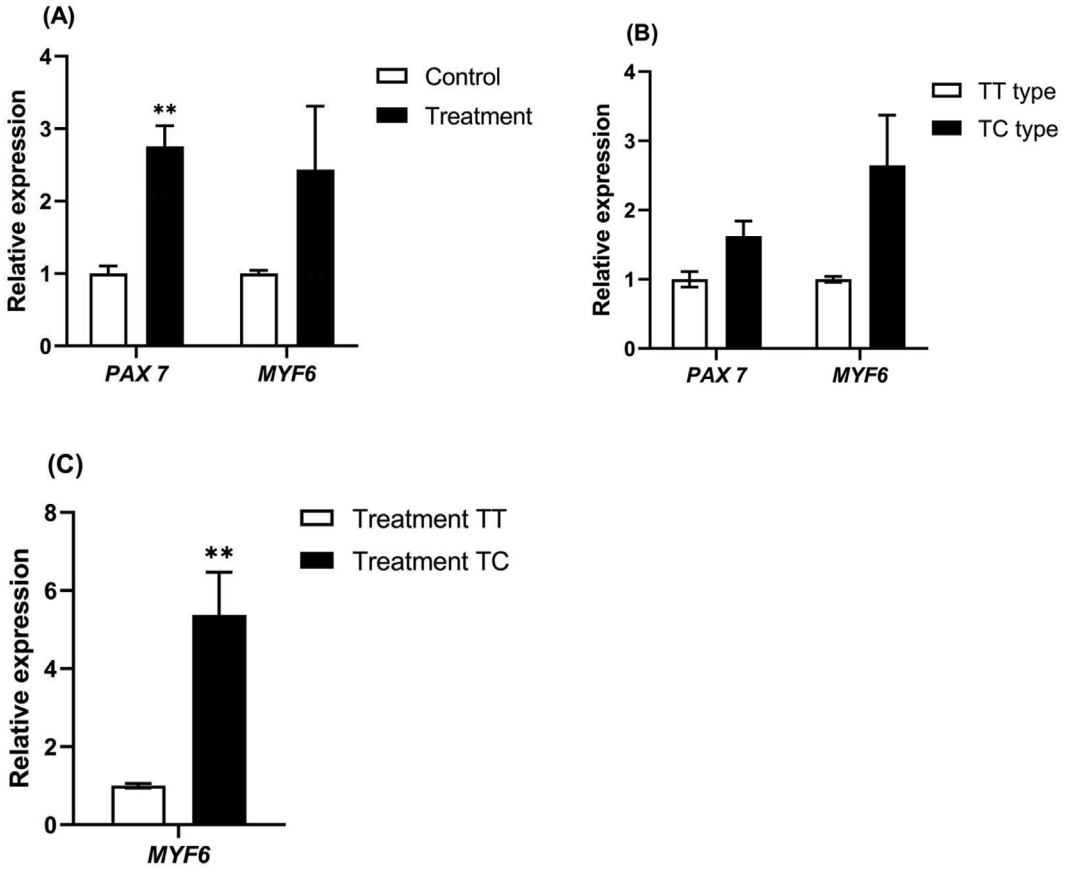

Fig. 3. Expression of target mRNA of longissimus dorsi muscle in weaned calves based on *ADH1C* genotype after vitamin A supplementation. (A) *PAX7* and *MYF6* mRNA expression in control and vitamin A treatment group. (B) *PAX7* and *MYF6* mRNA expression in TT and TC type of *ADH1C* genotype. (C) *MYF6* mRNA expression of TT and TC type of *ADH1C* genotype in vitamin A treatment group. *PAX7* = paired box 7; *MYF6* = myogenic factor 6. Vitamin A supplementation (40,000 IU/kg) in as-fed calves for 3 to 6 months of age after weaning. The quantitative real-time PCR values are expressed as fold changes after normalization against the control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*) mRNA. Data are expressed as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. *PAX7* × treatment × genotype: $P > 0.05$, *MYF6* × treatment × genotype: $P > 0.05$.

study, dietary vitamin A supplementation significantly increased serum vitamin A level in calves. Similarly, supplying 100 IU/kg BW of vitamin A to Shal sheep aged 4 to 6 months significantly increased plasma vitamin A compared to receiving 50 IU/kg BW (Asadian and Mezes, 1996). It suggested that although large amounts of vitamin A injection in one dose led to a rapid increase in blood vitamin A concentration in a short time, maintaining blood vitamin A levels stably at a high level requires consistent vitamin A administration orally (Peng et al., 2020a; Reddy and Mohanram, 1981). Also, we found that serum vitamin A concentration was significantly lower in TC calves than in TT calves after vitamin A supplementation. However, differences in serum vitamin A between *ADH1C* genotypes were not observed in previous studies of vitamin A restriction in Angus crossbred steers and Korean native steers (Peng et al., 2017; Ward et al., 2012). This result indicated that TC types exhibit higher conversion of vitamin A in Korean native claves.

To determine the effect of vitamin A supplementation on the metabolic status of calves, we also performed a metabolic profile test. Increases in serum AST and ALT concentrations are indicative of liver injury (Cebra et al., 1997; Kauppinen, 1984; Meyer, 1998). No difference was found in AST or ALT concentrations between control and vitamin A treatment groups in this study, which indirectly indicates that the liver was not adversely influenced by vitamin A supplementation. In addition, our previous study reported that serum NEFA concentration was significantly increased in vitamin A-restricted Korean native steers (Peng et al., 2019). In the current study, we found that vitamin A supplementation significantly reduced serum NEFA and TG concentrations, which indicated that vitamin A supplementation in cattle might be positively associated with lipid mobilization during subsequent fat induction. Moreover, serum AST concentration in TC calves at 6 months of age was significantly lower than that of TT type, which may be related to lower serum vitamin A levels in TC type. Accordingly, extra vitamin A supplementation was not associated with hepatic side effects. Meanwhile, the TC type may induce higher tolerance than the TT type.

It is known that ADH1 and ALDH1A1 enzymes play a major role in the conversion of retinol to RA (Molotkov and Duester, 2003; Molotkov et al., 2002). Krone et al. (2016) reported that the expression of ADH1C protein was significantly increased after the retinol in the liver was depleted. Further, our study found that vitamin A supplementation significantly reduced the *ADH1C* and *ALDH1A1* mRNA expression. In the current study, extra vitamin A supplementation might result in large amounts of retinol stored in the hepatic stellate cells in the form of retinyl esters, which decreased the amount of free retinol and consequently reduced the mobilization of vitamin A metabolic enzyme (Molotkov et al., 2004). A further study investigating liver metabolism following vitamin A supplementation is needed. In addition, the *ADH1C* mRNA expression was significantly higher in the TC type than in the TT type in the liver of Korean native calves after vitamin A

supplementation, but no difference was detected in *ALDH1A1* mRNA. Previous studies in Angus crossbred steers showed that the expression of the *ADH1C* mRNA and protein in the liver was higher in the TT type than the TC type (Krone et al., 2016; Ward et al., 2012). The findings suggested that the C allele of *ADH1C c.-64T > C* SNP eliminates the potential binding site motif of transcription factor CCAAT enhancer binding protein-α (*C/EPBα*), thereby reducing the expression of *ADH1C* mRNA (Ward et al., 2012). Besides, we speculated the existence of another mechanism regulating the metabolic activity of ADH1C enzyme to explain our current results. Moreover, Krone et al. (2016) inferred that increase in *ADH1C* mRNA expression translates into an increase in the production of ADH1C enzyme. In this study, the high level of *ADH1C* mRNA expression in TC type suggested TC type consumed more retinol, which was consistent with a significantly lower serum retinol concentration than the TT type. Therefore, these results implied that Korean native calves expressing TC genotype are more efficient at converting retinol to RA.

In the postnatal stage, muscle development is accomplished via hypertrophy of existing muscle fibers and activation of satellite cells (Bonnet et al., 2010). The satellite cells are located beneath the basal lamina, which express *PAX7* in the quiescent state (Seale et al., 2000; Zammit et al., 2006). An injection of 150,000 IU of vitamin A significantly upregulated the mRNA expression of *PAX7*, *MYF5*, *MYOD* and *MYF6* in the muscle tissue of neonatal Angus calves and significantly increased the number of satellite cells (Wang et al., 2018). Oral vitamin A supplementation of Korean native calves also enhanced the mRNA expression of *MYOD*, *MYF6*, and *MYOG* in longissimus dorsi muscle, without affecting the levels of *PAX7* and *MYF5* (Peng et al., 2020a). In the current study, additional vitamin A supplementation in calves upregulated the mRNA expression of *PAX7* in longissimus dorsi muscle, but had no significant effect on other myogenic genes. Our results implied that vitamin A supplementation may increase the storage of satellite cells, but did not convert more satellite cells into myoblasts for muscle development. Besides, we observed that *MYF6* mRNA expression was significantly higher in the TC type in than the TT type after vitamin A supplementation. Also, we found that the BW of TC type calves was 8.3 kg higher than in TT type at 6 months of age after vitamin A supplementation, without any statistically significant difference. It appeared that TC type calves converted more vitamin A to RA, thereby promoting muscle development at the genetic level. In the weaning calves, vitamin A supplementation regulated the preadipocyte and muscle fiber development related mRNA level and metabolic parameters (Peng et al., 2020a; 2020b). However, we found that the expression of mRNA associated with adipogenesis in weaned calves was not significantly changed by vitamin A supplementation. These results indicated that adipogenesis and muscle development in weaned calves are insensitive to vitamin A supplementation than neonates as reported in our previous study. Therefore, our results implied that vitamin A supplementation increases the storage of satellite cells in the muscle of weaned calves, thereby suggesting more gradient vitamin A treatment and experimental period is necessary in the future research.

5. Conclusion

In this study, dietary supplementation of extra vitamin A increased the storage of satellite cells in the muscle of weaned calves, which provides a basis for future muscle development. In addition, vitamin A supplementation downregulated the mRNA expression of vitamin A metabolic enzymes in the liver, suggesting the need for further studies to investigate changes in hepatic metabolism. Moreover, the TC type of *ADH1C* is associated with greater vitamin A metabolism than the TT type. Thus, vitamin A supplementation combined with *ADH1C c.-64T > C* genotype has a potential role in genetically manipulating the feed management program for beef production in Korean native calves.

Author contributions

Xue Cheng Jin: methodology, formal analysis, writing - original draft; **Dong Qiao Peng**: conceptualization, methodology, writing - review and editing; **Seong Jin Kim**: assistance in the management of calves; **Na Yeon Kim**: assistance in the management of calves; **Jalil Ghassemi Nejad**: writing - review and editing; **Danil Kim**: biopsy sampling; **Stephen B. Smith**: writing - grammatically correction and review; **Hong Gu Lee**: conceptualization, writing - review and editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix. supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.06.007.

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