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Original Research Article

Neonatal vitamin A but not retinoic acid administration increases intramuscular adipocyte number in sheep by promoting vascularization

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A B S T R A C T

This study investigated whether vitamin A (VA) administration during the neonatal stage could increase the number of intramuscular adipocytes in Hu sheep by promoting vascularity. A total of 56 newborn male Hu sheep were divided into four groups and received intramuscular injections of either 0, 7500 IU retinoic acid (RA), 7500 IU VA, or a combination of 7500 IU VA and 5 mg SU5416 (an angiogenic inhibitor), at 1, 7, 14, and 21 days of age. At 15 days of age, 6 sheep from each group were randomly selected and sacrificed for intramuscular adipogenic capacity analysis. The remaining 8 sheep in each group were raised until they were 8 months old. VA-treated sheep exhibited an increase in preadipocytes, elevated expression of adipogenic genes (CCAAT enhancer binding protein alpha [*CEBPA*] and CCAAT enhancer binding protein beta [*CEBPB*]) and angiogenic genes (vascular endothelial growth factor A [*VEGFA*]), and stromal vascular fraction cells in the longissimus dorsi (LD) muscle with enhanced adipogenic capacity ($P < 0.05$). These effects were entirely negated by SU5416. Upon slaughter, VA increased final weight, carcass weight, and average daily gain ($P < 0.05$) but did not affect feed intake at 21 to 32 weeks ($P = 0.824$). VA increased the number of intramuscular adipocytes in the LD and semitendinosus (ST) muscle ($P < 0.05$) without changing the adipocyte number of the omentum, perirenal and subcutaneous fats ($P > 0.05$). VA injections also increased intramuscular triglyceride (TG) content ($P = 0.016$) without changing the omentum fat weight or subcutaneous fat thickness ($P > 0.05$), but it did increase the perirenal fat weight ($P = 0.011$). Consistently, SU5416 mitigated the effects of VA on intramuscular TG content and adipocyte count, correlating with a decrease in vascularity. In contrast, RA injections didn't affect the intramuscular fat ($P = 0.744$) but reduced the TG content of the omentum and perirenal fat ($P < 0.05$). In conclusion, intramuscular injections of VA but not RA at the neonatal stage improved the growth performance of Hu sheep, increasing the number of intramuscular adipocytes and marbling by promoting angiogenesis.

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1. Introduction

Fat deposition affects both the meat quality and feed efficiency of livestock (Sillence, 2004). The deposition of visceral and subcutaneous fat reduces feed efficiency, as it requires more energy compared to muscle growth (Owens et al., 1995). Unlike visceral and subcutaneous fat, which is often inedible, intramuscular fat (IMF) improves meat juiciness and flavor, meeting consumer

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preferences (Realini et al., 2021). To produce marbled meat, producers feed animals with a high-energy diet at the finishing stage. However, this approach also increases the overall body fat mass, leading to a decrease in meat yield (McAllister et al., 2020; Wilkinson and Lee, 2018). Consequently, there's a growing focus on strategies that enhance IMF without increasing fat in other depots. In vitro studies indicate that intramuscular adipocytes in beef cattle display a preference for glucose over acetate for lipogenesis, in contrast to subcutaneous adipocytes (Smith and Crouse, 1984). Nonetheless, substituting a high-energy feed for an equivalent amount of energy from pasture did not elevate IMF in beef cattle (Greenwood et al., 2015). Evidence suggests that fat depots expand at a similar rate during the fattening stage (Pethick et al., 2004; Hocquette et al., 2010; Greenwood et al., 2015). To date, no effective nutritional strategy has been identified to target lipid deposition specifically in intramuscular adipocytes.

Although IMF is deposited lately and marbling is invisible at early life, the preadipocytes and adipocytes are formed postnatally and affects the potential of marbling development during growth (Hocquette et al., 2010). A previously suggested a method to increase IMF without increasing subcutaneous and visceral fat (Arana et al., 2008; Yu et al., 2022). The theory is based on the sequential formation and enlargement of adipocytes in subcutaneous, visceral, intermuscular, and IMF (Hausman et al., 2014). While de novo adipogenesis primarily occurs in the subcutaneous and visceral fat before birth in ruminant animals (Du et al., 2013), the total number of adipocytes in these fat depots remains largely constant after adolescence (Bonnet et al., 2010; Schoonmaker et al., 2004). In contrast, intramuscular adipogenesis occurs later and continues from birth until later in life (Bonnet et al., 2010). Thus, a nutritional strategy that promotes adipogenesis at the neonatal stage could increase the number of intramuscular adipocytes without affecting other fat depots.

Adipose tissues are highly vascularized (Nijhawans et al., 2020). Blood vessels play a crucial role in ensuring the functioning of adipose tissue and regulating the development and replenishment of adipocytes (Cao, 2007; Angueira et al., 2021). Blood vessels supply oxygen and nutrients to adipocytes, remove the metabolites, release growth factors to regulate adipogenesis and adipose tissue expansion (Bonnet et al., 2010; Crewe et al., 2018), and serve as a cellular reservoir which provides adipose progenitors for de novo adipogenesis (Cao, 2013). Thus, effective vascular development is required for adipose tissue development (Rupnick et al., 2002; Corvera et al., 2022). Intramuscular adipocytes are located near a blood capillary network (Chang et al., 2020). In beef cattle, the number of blood vessels significantly increases with marbling (Reddy et al., 1970). Wagyu cattle, a breed known for its marbling, have more vascular cells and a greater abundance of capillaries compared to leaner breeds (Wang et al., 2023). Therefore, nutrients that increase skeletal muscle vascularization may promote IMF development.

Vitamin A (VA) and its metabolite, retinoic acid (RA) have been well known to regulate adipogenesis and adipose tissue metabolism (Mercader et al., 2006; Wang et al., 2017c). RA promotes the commitment of stem cells into adipose progenitors (Dani et al., 1997; Wang et al., 2017a) but inhibits the terminal differentiation of white adipocytes and inhibits lipid accumulation (Schwarz et al., 1997; Berry et al., 2012; Wang et al., 2017c). RA also promotes the formation of brown adipocytes by upregulating PR domain containing 16 (Wang et al., 2017a) and enhances energy expenditure by upregulating thermogenic genes like uncoupling protein 1 (Mercader et al., 2006). As a result, administering VA or RA leads to energy dissipation (Berry and Noy, 2009) and weight loss in adult animals (Berry et al., 2012; Wang et al., 2017c). Consequently, VA is restricted for fattening beef cattle to increase lipid accumulation (Gorocica-Buenfil et al., 2007; Arnett et al., 2009; Ward et al., 2012). On the other hand, as a promoter of the vascular endothelial growth factor A, RA increases adipose progenitors by promoting tissue vascularization (Wang et al., 2017a). Leveraging this, a previous study treated Angus beef cattle with VA at birth and one month of age. As anticipated, intramuscular VA injection at the neonatal stage enhanced the intramuscular adipogenic potential of beef cattle and increased IMF content at slaughter (Harris et al., 2018; Yu et al., 2022). Subsequent studies performed by other groups confirmed the effects of VA (Maciel et al., 2022). Some studies also found that neonatal VA administration promoted muscular vascularization and increased the number of platelet derived growth factor receptor α-positive (PDGFRα^+) adipose progenitor cells in the skeletal muscle of beef cattle (Peng et al., 2020; Yu et al., 2022). In vitro studies also showed that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 (VEGFA/VEGFR2) signaling is required for the adipogenic-promoting effects of RA (Yu et al., 2022). However, whether VA increased beef cattle marbling by stimulating angiogenesis has not been confirmed in vivo. Previous studies have observed an increase in intramuscular preadipocytes and improved marbling in beef cattle, but the number of intramuscular adipocytes had not been quantified.

This study tested whether intramuscular VA/RA injection at the neonatal stage would increase the intramuscular adipogenic potential of Hu sheep. A group of animals was treated with VA and SU5416, an inhibitor of angiogenesis, simultaneously to verify the mediatory roles of angiogenesis in increasing intramuscular adipocytes.

2. Materials and methods

2.1. Animal ethics statement

Animal studies were performed according to a protocol (Approvement code: AW60604202-1-1) approved by the Institutional Animal Care and Use Committee at China Agricultural University.

2.2. Animals

Hu sheep ewes with similar body conditions were synchronized and inseminated with semen from one Dorper ram. All ewes were at their third parity. Three pregnant ewes per stall had free access to food and water and the diet was formulated according to National Research Council (NRC, 2007). The number of fetuses was determined at 35 days of gestation using an ultrasound monitor. In this study, only twin lambs were used for further experiments. At birth, one lamb from each pair of male twins was selected. A total of 56 lambs weighing 3.45 ± 0.52 kg were randomly assigned into four groups. The lambs were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU VA palmitate (PHR1235, Sigma, Milwaukee, US) with corn oil as solvent, 7500 IU RA (PHR1187, all-trans-retinoic acid, Sigma, Milwaukee, US) with corn oil as solvent, and a mixture of 7500 IU VA-5 mg SU5416 (an inhibitor of the VEGF receptor, S8442, Sigma, Milwaukee, US) with corn oil as solvent. The lambs were injected once a week at a fixed time point for 3 weeks and managed in pairs with ewes. The dosages of VA and RA were determined according to our previous research on beef cattle (Yu et al., 2022) with adjustment made based on differences in body weight.

The ambient temperature of the sheep house was maintained at around 10 °C and well-ventilated. Lambs were vaccinated against combined ovine/caprine braxy, struck, lamb dysentery, and enterotoxaemia (Harbin Pharmaceutical Group Bio-vaccine Co., Ltd., Harbin, China) on 10 days of age and then vaccinated against sheep

pox (Harbin Pharmaceutical Group Bio-vaccine Co., Ltd., Harbin, China), Peste des petits ruminants (Tecon Biological Co., Ltd., Xinjiang, China) and foot-and-mouth disease (Inner Mongolia Bigvet Biotech Co., Ltd., Inner Mongolia, China) on 28, 47, and 49 days of age, respectively. All lambs were weaned at 12 weeks of age and then transferred to the finishing houses for further rearing. Feed is rationed according to the stage of development, feed twice a day and residual intake was measured. After weaning, lambs were fed backgrounding diets consisting of concentrate and hay (concentrate:roughage = 5:5) for 55 days and then transitioned to the finishing diet with free access to clean water and salt blocks. During the finishing period, sheep received finishing diets consisting of concentrate and hay (concentrate:roughage = 8:2). In addition, the concentrate feed is a commercial diet (Shanxi Guannong Science and Technology Co., Ltd., China) (composition of nutrient levels of the concentrate feed as shown in Table 1), grass hay (peanut seedlings) for sheep growth was added during the finishing period (nutrient levels of the grass hay as shown in Table 2).

Table 2
Nutrient levels of the grass hay (DM basis, %).

Item	Content
DM	87.84
CP	12.32
EE	2.30
Ash	11.26
NDF	63.47
ADF	40.01

DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber.

2.3. Feed nutrients analysis

The feed samples were dried at 55 °C for 72 h, then crushed and passed through a 1-mm sieve (Method 950.02). The feed nutrients were determined according to AOAC (1990). For commercial diet and grass hay, the content of dry matter (DM) was determined after the samples were dried in an air-forced oven at 135 °C for 2 h (method 930.15). The nitrogen content was measured by the Kjeldahl method (method 955.04), and the crude protein (CP) content was calculated by multiplying the nitrogen content by 6.25 (method 954.01). The ether extract (EE) content was measured by a Soxhlet apparatus (Ankom TX15, ANKOM Technology, Macedon, NY, USA) (method 920.39). The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by the Van Soest method (Van Soest et al., 1991). The ash concentrations were determined by placing a weighed sample in a muffle furnace (SX2-12–10, Lichen Instrument Technology Co., Ltd., Shanghai, China) at 600 °C for 2 h (method 942.05). A nylon bag method was used to determine rumen undegraded protein (RUP) (AFRC–AGRICULTURAL, 1992). Calcium was determined through a reaction with ammonium oxalate and titration using potassium permanganate (method 927.02). Phosphorus was determined by alkalimetric ammonium molybdophosphate method (method 964.06). Net energy of growth (NEg) and metabolizable energy (ME) were estimated according to National Research Council (NRC, 2007).

Table 1
Compositions and nutrient levels of the concentrate feed at backgrounding and finishing stages of lambs (DM basis, %).

Item	Backgrounding	Finishing
Ingredients		
Corn	60.00	75.00
Wheat bran	11.00	
Soybean meal	15.00	8.00
Soya bean cake	8.00	10.00
Premix (4 %)[1]	5.00	5.00
Baking soda	1.00	2.00
Total	100.00	100.00
Nutrient levels		
DM	87.4	87.3
CP	16.72	13.64
RUP	40.3	44.1
NEg, mcal/kg	1.30	1.32
ME, mcal/kg	2.83	2.85
NDF	16.6	13.9
ADF	6.1	5.1
EE	3.8	3.9
Ca	1.14	1.12
P	0.49	0.43
Ca: P	2.32	2.6

DM = dry matter; CP = crude protein; RUP = rumen undegraded protein; NEg =, net energy of growth; ME = metabolizable energy; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract.

[1] Supplied the following per kilogram premix: vitamin A, 2.5 mg; vitamin D_3, 1 mg; vitamin E, 30 mg; niacin, 10 mg; Ca-D-pantothenate, 5 mg; riboflavin, 2 mg; vitamin B_{12}, 5 mg; iron, 60 mg; copper, 25 mg; zinc, 50 mg; manganese, 25 mg; selenium, 0.2 mg; I, 0.2 mg.

2.4. Sample collection and analysis

At 15 days of age, 6 sheep were randomly selected from each group and slaughtered for sample collection. The other sheep were slaughtered at 8 months of age to collect omentum fat, perirenal fat, subcutaneous (back) fat, longissimus dorsi (LD) muscle and semitendinosus (ST) muscle. All sheep were fasted for 12 h before slaughter. All tissue samples were divided into three parts, one for histological analysis, one sample was stored in liquid nitrogen for RNA analysis, and one was used for stromal vascular fraction (SVF) cell isolation. The grade rule (GR) value is the thickness of adipose tissue measured with a caliper at 11 cm from the midline between the 12th and 13th ribs on the dorsal surface of the carcass (Xiang et al., 2022).

2.5. Cell culture and differentiation

Muscle samples collected at 15 days of age were washed in prechilled phosphate buffer (PBS) containing 1 × penicillin-streptomycin (C0222, Beyotime, China) and transported to the laboratory for cell isolation. All laboratory supplies were sterilized, muscle samples were trimmed with scissors, and a small portion was cut and digested in a digestion buffer (0.75 IU/mL collagenase D, 17100017, Gibco, USA; 1.0 IU/mL collagenase II, 17101015, Gibco, USA) at 37 °C for about 30 min. The tissue digest was filtered through a 40 μm cell filter sieve (352340, Corning, USA) and the filtrate was centrifuged at 500 × *g* for 5 min. The cell precipitate was collected and added to a DMEM medium containing 10% fetal bovine serum (FBS, C0252, Beyotime, China) and incubated in a 37 °C, 5% CO_2 incubator.

The adipogenic capacity was assessed using our previously developed 3D culture model. Briefly, the cell spheres were formed in hanging drops and transferred to a Matrigel (356255, Corning, USA) -coated 96-well plate. The culture was continued in the endothelial basal media-2 (EBM-2, CC-3156, Lonza, Switzerland) medium containing 10% FBS and 1 × penicillin-streptomycin for 3 days to allow the cell balls to grow adherently. Then adipogenesis was induced with insulin (2 μg/mL, P3376–100IU, Beyotime, China), dexamethasone (1 μg/mL, ST1254-1g, Beyotime, China), and isobutyl methylxanthine (27.8 μg/mL, SC0195–5 mg, Beyotime, China) for 3 days, followed by 3 days of insulin treatment (2 μg/mL).

2.6. Tissue processing and histological examination

Fresh adipose and muscle tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated, paraffin-embedded, and sectioned. Tissue slides were stained with hematoxylin-eosin for morphological analysis. For immunohistochemical staining, tissue sections of the LD muscle of 15-day-old lambs were subjected to antigen repair using 0.1 mol/L sodium citrate buffer (pH 6.0) by applying microwave heating at 98 °C for 20 min and cooling at room temperature. Tissue sections were incubated with antigen closure solution (10% goat serum, 0.25% Triton X-100, dissolved in PBS) at room temperature for 1 h, followed by incubation with platelet endothelial cell adhesion molecule-1 (CD31, ab119339, 1:200, Abcam, US) and PDGFRα (AF7704, 1:200, Beyotime, China) at 4 °C for 12 h. The corresponding secondary antibodies were incubated at room temperature for 1 h. Finally, the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL, C1005, Beyotime, China) for 3 min and sealed. Immunofluorescence imaging was performed under an EVOS fluorescence microscope (AMEX1200, Thermo Fisher Scientific, USA). Among them, CD31 and PDGFRα were used as markers for endothelial cells and adipose progenitor cells, respectively.

2.7. Triglyceride (TG) assay

Total lipids from fat and muscle were extracted using Folch's method (Folch et al., 1957). In detail, chip 30 to 50 mg of tissue and place into 1 mL of 2:1 chloroform: methanol (v/v) in a homogenizer tube. The samples were completely homogenized and shaken overnight at 4 °C. The next day, the tube is spun at 9500 × *g* for 10 min at 4 °C, and then 800 μL of supernatant is transferred to a new centrifuge tube, taking care not to touch the pellet. Add 0.2 mL of a 0.9% sodium chloride solution to each tube, cap, and vortex. The liquid will separate into 2 phases with the salt solution and methanol on top and the chloroform on the bottom. Spin again at 4 °C at 800 × *g* for 10 min and remove the supernatant and any particulate matter floating on top of the chloroform layer. Take a 400 μL sample of the bottom chloroform and then transfer it to a new tube. Put all the tubes containing the chloroform sample into the freeze dryer and evaporate until completely dry. Add American Chemical Society (ACS) grade 2-propanol to each tube (0.15 mL for muscle and 2 mL for adipose), vortex at high speed for 1 min. Then transfer the clean supernatant to another microfuge tube for analysis. The tissue TG content was determined using the TG assay kit (A110-1-1, Nanjing Jiancheng Bioengineering Institute, China).

2.8. Quantitative adipocyte numbers

The diameter of adipocytes was analyzed using Image J for each animal (Parlee et al., 2014). The content of adipose tissue TG was analyzed. We assume that each adipocyte is a TG-filled sphere, and the number of adipocytes is obtained by dividing the total tissue TG content by the TG content of each adipocyte. The formula for calculating the number of adipocytes is as follows:

$$N = \text{tissue weight} \times \frac{\text{M (TG)} \times \text{TG content}}{\rho\ (\text{TG}) \times \frac{4\pi}{3} \sum pi \left(\frac{di}{2}\right)^3}$$

where N is the number of adipocytes (subcutaneous (back) fat and IMF of LD muscle not multiplied by tissue weight); pi is frequency of different adipocyte diameter intervals; di is median values of different adipocyte diameter intervals; ρ (TG) = 0.9 g/cm^3, M (TG) = 639.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from adipose and muscle tissues and cells using TRIzol reagent (R0016, Beyotime, China) and cDNA was synthesized using a cDNA synthesis kit (D7168M, Beyotime, China). qRT-PCR was performed using SYBR green qRT-PCR kit (Q321, Vazyme, China) and CFX RT-PCR detection system (10,005,604, Bio-Rad, USA). The mRNA relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ and standardized to housekeeping genes (18S rRNA). The primer sequences are shown in Table 3.

2.10. Western blot analysis

Approximately 50 mg adipose samples were homogenized in 500 μL lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris–HCl, pH 8.0, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.5% NP-40, 1 mmol/L benzylsulfonyl fluoride, 10 mmol/L Na_3VO_4, and 1 mmol/L NaF) using a bead homogenizer. The homogenate was centrifuged at 12, 000 × *g* for 15 min at 4 °C. The upper phase lipid and sediment were discarded, and this step was repeated once. The protein concentration was determined using a BCA assay kit (P0011, Beyotime, China) and the total protein were denatured with a loading buffer containing 1% beta-mercaptoethanol at 95 °C for 5 min. Sample proteins were separated in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride membrane at 100 V for 90 min. Membranes were blocked with 5% bovine serum albumin (BSA) in TBST (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.05% Tween 20) for 2 h and subsequently incubated with primary antibodies against acetyl-CoA carboxylase alpha (ACC1, Abclonal, A19627, 1:1000, China), hormone-sensitive lipase (HSL, Abclonal, A15686, 1:1000, China), phospho-ACC1-S79 (p-ACC1, Abclonal, AP0298, 1:1000, China), phospho-HSL-S660 (p-HSL, Abclonal, Ap0853, 1:1000, China) or β-Tubulin (Beyotime, AF1216, 1:1000, China) at 4 °C overnight, then incubated with secondary antibody against HRP-labeled Goat Anti-Rabbit immunoglobulin G (IgG) (H + L) (Beyotime, A0208, 1:3000, China) at room temperature for 1 h. Protein bands were visualized using an ECL kit (P0018FS, Beyotime, China), imaged using a Tanon Imaging System (Tanon 5200, Tanon, China) and analyzed using Image J software (National Institutes of Health, Bethesda, USA).

2.11. Statistical analysis

The experiment was conducted in a completely randomized design. All data were found to be normally distributed and were analyzed by one-way ANOVA, with the following mathematical model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where Y_{ij} represents the value of the dependent variable for the jth observation under the ith treatment condition; μ is the population mean; α_i denotes the fixed effect of the ith treatment condition; ε_{ij} represents the random error associated with the jth observation under the ith treatment condition, which is assumed to be independently and identically distributed (i.i.d.) with a mean of zero and constant variance (σ^2). The Bonferroni test was performed when the overall effect was found to be significant. GraphPad Prism 8 was used for all statistical analyses. All data were presented as mean and standard error. Declared significant at $P < 0.05$.

Table 3
Primer sequences used for quantitative real-time PCR analyses.

Gene name	Accession number	Direction	Sequence (5′ to 3′)
ZFP423	XM_015106130.1	Forward	CTTCTTCTGCAACCAGTGCTCCAT
		Reverse	GTTCTTGTGGTTCTCCTTGATGTGCT
PPARG	NM_001100921.1	Forward	GCCGGGTCTGTGGGGATAAA
		Reverse	CGCCCAAACCTGATGGCATT
CEBPA	NM_001308574.1	Forward	ATCAGCGCCTACATCGACCC
		Reverse	GCCCGGGTAGTCAAAGTCGT
CEBPB	XM_012129601.2	Forward	AGCAAGGCCAAGAAGACGGT
		Reverse	GAACAAGTTCCGCAGGGTGC
VEGFA	NM_001025110.1	Forward	TCACCAAAGCCAGCACATAGGA
		Reverse	GCCTCGGCTTGTCACATTTTTC
VEGFR1	XM_015098156.3	Forward	AAGGAGATACGCGACTTCCTCTG
		Reverse	CCGCATCCTCATCCCATCCTT
VEGFR2	NM_001278565.1	Forward	TCTGCCTACCTCACCTGTTTCC
		Reverse	TCAGTCCACTAAAAGATGGGGC
LPL	NM_001009394.1	Forward	GAAGACTCGTTCTCAGATGCCTTAC
		Reverse	TCTCAGCCACAGTGCCATACAG
GLUT4	XM_027974995.3	Forward	GGTTCTTCATTGGTGCCTACTCAG
		Reverse	CGATTAGGATGCCAGTGACGATG
FASN	XM_027974304.3	Forward	AGATGAAGGTGGTAGAGGTGCTAG
		Reverse	TGGCGGTCAGTGGCTATGTAG
ACACA	NM_001009256.1	Forward	GACTTCACGCACCTTCTTCTACTG
		Reverse	TTCCCTCCACCTCCACAAACC
HSL	NM_001128154.1	Forward	AACAGCAGCGACACAACAGAC
		Reverse	GCCTCAGACACTTCAGATTCATCC
ATGL	NM_001308576.1	Forward	GGCTTCCTCGGCGTCTACC
		Reverse	GATGTGCGTGGCGTTGGC
DGAT1	NM_001110164.1	Forward	GGACACAGACAAGGACGGAGAC
		Reverse	ATCAGCATCACCACACACCAATTC
DGAT2	XM_027979550.3	Forward	GATGTCTGTGGTGATGGTGAAGTC
		Reverse	TGGACGGAGGAGCCTGGTAG
18S rRNA	NR_003278.3	Forward	CCTGCGGCTTAATTTGACTC
		Reverse	AACTAAGAACGGCCATGCAC

ZFP423 = zinc finger protein 423; *PPARG* = peroxisome proliferator activated receptor gamma; *CEBPA* = CCAAT enhancer binding protein alpha; *CEBPB* = CCAAT enhancer binding protein beta; *VEGFA* = vascular endothelial growth factor A; *VEGFR1* = vascular endothelial growth factor receptor 1; *VEGFR2* = vascular endothelial growth factor receptor 2; *LPL* = lipoprotein lipase; *GLUT4* = facilitated glucose transporter 4; *FASN* = fatty acid synthase; *ACACA* = acetyl-CoA carboxylase alpha; *HSL* = hormone-sensitive lipase; *ATGL* = adipose triglyceride lipase; *DGAT1* = diacylglycerol O-acyltransferase 1; *DGAT2* = diacylglycerol O-acyltransferase 2.

3. Results

3.1. Neonatal VA administration increases vascularization and the adipogenic potential of LD muscle

VA-injected sheep had more PDGFRA adipose progenitors ($P = 0.037$) in the LD muscle at the age of 15 days (Fig. 1A). However, when angiogenesis was suppressed by SU5416, the ability of VA to augment adipose progenitors was negated. Correspondingly, the injection of VA led to an upregulation of the adipogenic genes CCAAT enhancer binding protein alpha (*CEBPA)* and CCAAT enhancer binding protein beta (*CEBPB*) ($P < 0.05$; Fig. 1B), as well as the angiogenic gene *VEGFA* in the LD muscle of 15-day-old sheep ($P = 0.003$, Fig. 1C). The increase of *VEGFA, CEBPA*, and *CEBPB* gene relative expression in the LD muscle was prevented by SU5416 ($P < 0.05$; Fig. 1B and C). In vitro adipogenesis demonstrated that SVF cells, isolated from the LD muscle of 15-day-old sheep injected with VA, significantly promoted lipid deposition compared to those from other groups ($P = 0.014$), and this effect was mitigated by SU5416 ($P = 0.005$) (Fig. 1D). These findings suggest that VA increases the number intramuscular adipose progenitors and boosts the adipogenic potential of LD muscle by promoting angiogenesis.

3.2. Neonatal VA administration increases IMF content and the number of intramuscular adipocytes of sheep at harvest

In accordance with the observed increase in adipose progenitors, VA significantly increased the TG content in both LD and ST muscle (Figs. S1A and B), and also increased the total TG content in the ST muscle at harvest ($P = 0.011$) (Table 4). This study further investigated the diameters of intramuscular adipocytes in the LD and ST muscles of sheep at harvest. It was found that neonatal VA injection did not alter the intramuscular adipocytes diameter in the LD muscle and ST muscle ($P > 0.05$; Table 4). This study further calculated the number of adipocytes in different fat tissues as described in the method section. In line with the increase in preadipocytes observed at the age of 15 days, VA-treated sheep had a significantly higher number of intramuscular adipocytes in the LD and ST muscles ($P < 0.05$; Table 4). However, SU5416 injection mitigated the effects of VA ($P = 0.021$) (Table 4). These findings suggest that neonatal VA injection increases the number of intramuscular adipocytes and the content of IMF by promoting angiogenesis.

3.3. Neonatal VA injection on overall fatness of sheep

Intramuscular injection of VA and RA at the neonatal stage led to an increase in the final weight, carcass weight, and average daily gain ($P < 0.05$; Table 5). Feed intake at 21 to 32 weeks of VA and RA-treated animals was not statistically significant ($P = 0.824$) (Table 5). Regardless of whether SU5416 was administered or not, VA injection significantly increased both the perirenal fat weight ($P = 0.011$) (Table 5) and adipocyte diameter of perirenal fat at harvest ($P = 0.047$) (Fig. S1C and Table 4). However, no differences were observed in the omentum fat weight and GR value ($P > 0.05$; Table 5), and adipocyte diameter of omentum fat and subcutaneous (back) fat ($P > 0.05$; Figs. S1D and E, Table 4). Moreover, RA significantly reduced the TG content in the perirenal fat ($P = 0.040$)

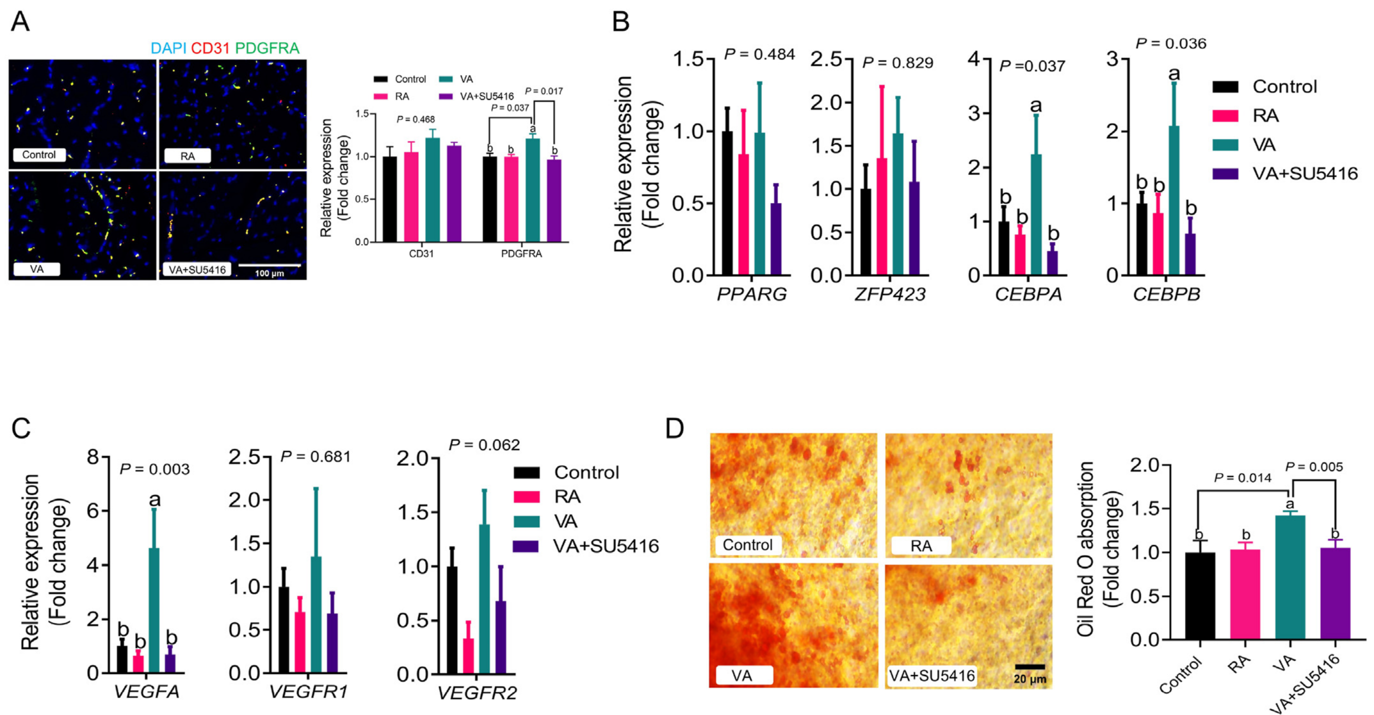

Fig. 1. Neonatal injection of vitamin A affects intramuscular fat deposition potential. (A) Representative immunofluorescence staining images of PDGFRA and CD31 from LD muscle of 15-day-old sheep, along with the relative expression levels of CD31 and PDGFRA. (B) Adipogenic genes expression in the LD muscle of 15-day-old sheep. (C) Angiogenic genes expression in the LD muscle of 15-day-old sheep. (D) Representative images of Oil Red O stained intramuscular SVF cells formed adipocytes and quantification of Oil Red O absorption. The sheep were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU vitamin A palmitate (VA) with corn oil as solvent, 7500 IU all-trans-retinoic acid (RA) with corn oil as solvent, and a mixture of 7500 IU VA-5 mg SU5416 (an inhibitor of the soluble vascular endothelial growth factor receptor) with corn oil as solvent. DAPI = 4′,6-diamidino-2-phenylindole; PDGFRA = platelet derived growth factor receptor α-positive; CD31 = platelet endothelial cell adhesion molecule-1; LD = longissimus dorsi; SVF = stromal vascular fraction; *PPARG* = peroxisome proliferator activated receptor gamma; *ZFP423* = zinc finger protein 423; *CEBPA* = CCAAT enhancer binding protein alpha; *CEBPB* = CCAAT enhancer binding protein beta; *VEGFA* = vascular endothelial growth factor a; *VEGFR1* = vascular endothelial growth factor receptor 1; *VEGFR2* = vascular endothelial growth factor receptor 2. Data were shown as mean ± SEM; $n = 6$ in each group. [a, b] Mean with different letters are significantly different from each other ($P < 0.05$).

Table 4
Neonatal VA injection on TG content, adipocyte diameter, and adipocyte number of sheep[1].

Item	Control	RA	VA	VA + SU5416	SEM	*P*-value
TG content in LD muscle, μmol/g	22.71^{b}	24.89^{b}	41.43^{a}	26.95^{b}	14.606	0.016
TG content in ST muscle, μmol/g	7.65^{b}	6.83^{b}	16.69^{a}	10.20^{ab}	6.375	0.023
Total TG content in ST muscle, g	0.76^{b}	0.73^{b}	1.93^{a}	1.11^{ab}	0.746	0.011
TG content in perirenal fat, μmol/g	636.36^{a}	487.49^{b}	560.19^{ab}	522.87^{ab}	104.222	0.040
Total TG content in perirenal fat, g	93.53^{b}	93.84^{b}	151.24^{a}	131.14^{a}	31.582	<0.001
Non-TG content in perirenal fat, g	136.47^{c}	207.41^{b}	271.26^{a}	261.36^{a}	55.356	<0.001
TG content in omentum fat, μmol/g	316.35^{a}	226.12^{b}	217.65^{b}	263.51^{ab}	55.246	0.003
Total TG content in omentum fat, g	155.66	137.99	161.51	174.91	27.255	0.137
Non-TG content in omentum, g	614.34^{d}	817.01^{c}	999.75^{a}	863.84^{b}	140.309	<0.001
TG content in subcutaneous (back) fat, μmol/g	410.88	351.20	396.24	369.47	68.107	0.483
Adipocyte diameter of perirenal fat, μm	40.94^{b}	42.46^{ab}	49.02^{a}	52.83^{a}	7.332	0.047
Adipocyte diameter of omentum fat, μm	55.75	53.19	58.18	58.72	7.988	0.711
Adipocyte diameter of subcutaneous (back) fat, μm	52.20	48.18	50.28	50.58	5.987	0.754
Adipocyte diameter of LD muscle, μm	31.36	34.89	30.13	29.09	5.689	0.424
Adipocyte diameter of ST muscle, μm	21.70	21.51	27.48	24.35	3.598	0.107
Adipocyte numbers of LD muscle (per g tissue)	7.00×10^{5b}	7.10×10^{5b}	1.21×10^{6a}	7.94×10^{5b}	4.239×10^{5}	0.026
Adipocyte numbers of ST muscle (whole tissue)	6.15×10^{7b}	8.13×10^{7ab}	1.19×10^{8a}	5.80×10^{7b}	4.245×10^{7}	0.039
Adipocyte numbers of omentum fat (whole tissue)	1.46×10^{9}	1.35×10^{9}	1.20×10^{9}	1.34×10^{9}	2.180×10^{8}	0.266
Adipocyte numbers of perirenal fat (whole tissue)	1.94×10^{9}	1.74×10^{9}	1.94×10^{9}	1.57×10^{9}	3.308×10^{8}	0.183
Adipocyte numbers of subcutaneous (back) fat (per g tissue)	2.56×10^{6}	2.71×10^{6}	2.65×10^{6}	2.46×10^{6}	4.600×10^{5}	0.833

LD = longissimus dorsi; ST = semitendinosus; TG = triglyceride.
[a-d] Means with different letters are significantly different from each other ($P < 0.05$).
[1] The sheep were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU, vitamin A palmitate (VA) with corn oil as solvent, 7500 IU, all-trans-retinoic acid (RA) with corn oil as solvent, and a mixture of 7500 IU, VA-5 mg SU5416 (an inhibitor of the soluble vascular endothelial growth factor receptor) with corn oil as solvent. Data were shown as mean with SEM provided, $n = 8$ in each group.

(Table 4). Although the total TG content in the perirenal fat increased by 61.70% ($P < 0.001$), the non-TG content of the perirenal in the VA treated group increased by 98.77% ($P < 0.001$) (Table 4), indicating that the increase in the perirenal fat is not just caused by fat accumulation. Both RA and VA significantly reduced the TG content of the omentum fat ($P = 0.003$), while the total TG content in omentum fat was not changed significantly ($P = 0.137$), the non-TG content of the omentum fat in the RA and VA treated group significantly increased ($P < 0.001$) (Table 4). Lastly, TG content in subcutaneous fat remained unchanged ($P = 0.483$) (Table 4). In accord with our cognition that most subcutaneous and visceral adipocytes are already formed after birth, neither RA nor VA injection at the neonatal stage altered the number of adipocytes in the omentum, perirenal, or subcutaneous (back) fat ($P > 0.05$; Table 4).

Similarly, the relative expression of adipogenic genes (including peroxisome proliferator activated receptor gamma, zinc finger protein 423, *CEBPA* and *CEBPB*) and angiogenic genes (including *VEGFA*, vascular endothelial growth factor receptor 1 [*VEGFR1*] and *VEGFR2*) in the omentum, perirenal or subcutaneous fat was not affected by either RA or VA injection ($P > 0.05$), with the relative exception of VA upregulating *CEBPA* in the subcutaneous fat ($P = 0.031$) (Table 6). Thus, this study further investigated whether VA and RA injections affected lipid metabolism in the visceral and subcutaneous fat depots at 15 days of age. RA significantly reduced the protein expression of the lipogenic enzyme ACC1 in the perirenal ($P = 0.048$) (Fig. 2A). VA also reduced the ACC1 protein expression in the perirenal and omentum fat tissues ($P < 0.05$; Fig. 2A and B). Neither VA nor RA injections affected the expression of lipogenic and lipolytic genes in the omentum or subcutaneous fat tissues ($P > 0.05$). However, VA inhibited the expression of both the lipogenic gene diacylglycerol O-acyltransferase 1 and the lipolytic gene adipose triglyceride lipase in the perirenal fat ($P < 0.05$; Table 6).

4. Discussion

IMF is highly correlated with the flavor, tenderness, and juiciness of meat (Scollan et al., 2017). Sheep meat with an IMF content of 5% or more is preferred by consumers (Pannier et al., 2018), while less than 3% negatively impacted dietary scores (Watkins et al., 2013). In ruminants, the late gestation, neonatal period, and early weaning (1–2 months of age) are the most effective periods for increasing the number of adipocytes in the muscle through nutritional regulation, as there are sufficient pluripotent cells available during these times (Jennings et al., 2016; Zhao et al., 2023). However, after weaning, due to the depletion of pluripotent cells, the increase in marbling primarily comes from the increase in adipocyte size (Du et al., 2010; Wang et al., 2016).

Previous studies have shown that administering VA to beef cattle via oral supplementation or intramuscular injection at the neonatal stage increases the number of preadipocytes and improves beef cattle marbling (Peng et al., 2020; Maciel et al., 2022; Yu et al., 2022). Unlike the intramuscular adipocytes that form later, adipocytes in subcutaneous and visceral fat are mainly formed before or around birth (Cianzio et al., 1985; Schoonmaker et al., 2004; Bonnet et al., 2010). Consistent with this, neonatal VA injection in cattle (Yu et al., 2022) and sheep did not change the number of adipocytes in other fat depots. Although VA and RA are known to enhance lipid oxidation and prevent lipid accumulation in mature adipocytes (Berry and Noy, 2009; Berry et al., 2012; Wang and Du, 2023), VA injected at the neonatal stage will degrade quickly and the serum VA level will return to normal before the finishing stage (Gannon et al., 2021). Interestingly, although RA is the major retinoid that regulates adipogenesis (Wang et al., 2017b; Wang and Du, 2023), in the current study, RA didn't alter the number of intramuscular adipocytes in sheep. This could be due to the instability of RA.

Although it is widely recognized that an increase in the number of adipocytes enhances the ability of adipose tissue to store lipids (Wang et al., 2016; Berger and Géloën, 2023), the adipocyte number in a certain tissue is rarely measured. To estimate the number of adipocytes in muscle and fat tissues, this study assumed that all adipocytes were spherical and filled with TG, and the number of adipocytes was calculated by dividing the amount of TG in the tissue by the amount of TG in adipocytes. Although this is still a rough estimate, it provides data to directly demonstrate that intramuscular VA injection at the neonatal stage increased the number of intramuscular adipocytes.

In rodents, RA increases blood vessel density, which, in turn, increases the number of adipose progenitors located surrounding the vascular vessels (Wang et al., 2017a,b; Wang and Du, 2023). While previous research demonstrated that intramuscular VA injection in beef cattle at birth and 1 month of age increased blood vessel density and preadipocyte numbers in skeletal muscle (Yu et al., 2022), there was no in vivo evidence showing that VA enhances marbling by promoting angiogenesis. This study found that VA administration to Hu sheep during the first two weeks after birth increased the number of intramuscular adipocytes. More importantly, by injection of SU5416, an inhibitor of the VEGF receptor (Fong et al., 1999), this study demonstrated that VA increases preadipocyte numbers and improves sheep marbling by promoting

Table 5
Neonatal VA injection on animal growth performance[1].

Item	Control	RA	VA	VA + SU5416	SEM	*P*-value
Birth weight, kg	3.76	3.80	3.74	3.74	0.340	0.981
Weaning weight, kg	20.64	24.34	23.99	22.67	3.162	0.081
Final weight, kg	47.56[b]	56.46[a]	56.93[a]	54.51[ab]	6.572	0.011
Average daily gain, kg/d	0.20[b]	0.24[a]	0.24[a]	0.23[ab]	0.029	0.012
Carcass weight, kg	25.01[b]	30.13[a]	30.94[a]	29.58[ab]	3.924	0.007
Perirenal fat weight, kg	0.23[b]	0.30[ab]	0.42[a]	0.39[a]	0.134	0.011
Omentum fat weight, kg	0.77	0.96	1.16	1.04	0.413	0.307
GR value, mm	21.41	23.28	23.1	22.65	3.998	0.811
Semitendinosus muscle weight, kg	0.16	0.17	0.18	0.17	0.029	0.861
12–20 week feed intake, kg/d	0.85	0.95	0.94	0.91	0.283	0.965
21–32 week feed intake, kg/d	1.21	1.35	1.36	1.31	0.264	0.824

[a,b] Means with different letters are significantly different from each other ($P < 0.05$).
[1] The sheep were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU, vitamin A palmitate (VA) with corn oil as solvent, 7500 IU, all-trans-retinoic acid (RA) with corn oil as solvent, and a mixture of 7500 IU, VA-5 mg SU5416 (an inhibitor of the soluble vascular endothelial growth factor receptor) with corn oil as solvent. Data were shown as mean with SEM provided, $n = 8$ in each group.

Table 6
Results of mRNA expression in different regions of adipose tissue from 15-day-old sheep (fold change)[1].

Item		Control	RA	VA	VA + SU5416	SEM	*P*-value
Omentum fat							
Lipogenesis	*DGAT1*	1.000	0.567	1.567	1.096	0.3550	0.078
	DGAT2	1.000	0.677	0.430	0.384	0.2447	0.174
	FASN	1.000	0.489	0.191	0.689	0.2946	0.151
	ACACA	1.000	0.559	0.365	0.915	0.2588	0.385
	GLUT4	1.000	0.833	1.938	0.554	0.5196	0.098
Lipolysis	*ATGL*	1.000	0.570	0.559	0.302	0.2504	0.152
	HSL	1.000	1.458	1.932	1.289	0.3380	0.753
	LPL	1.000	0.708	0.627	0.632	0.1525	0.790
Adipogenesis	*PPARG*	1.000	1.166	0.845	0.188	1.2882	0.651
	ZFP423	1.000	2.377	1.609	0.470	1.2695	0.095
	CEBPA	1.000	1.574	0.979	0.610	0.9602	0.107
	CEBPB	1.000	2.427	2.575	1.423	1.1121	0.387
Angiogenesis	*VEGFA*	1.000	0.649	1.520	0.374	2.7822	0.185
	VEGFR1	1.000	1.491	2.210	1.033	0.8001	0.204
	VEGFR2	1.000	1.224	0.821	1.083	0.7632	0.908
Perirenal fat							
Lipogenesis	*DGAT1*	1.000[a]	0.297[ab]	0.277[b]	0.488[ab]	0.2916	0.030
	DGAT2	1.000	0.222	0.335	0.858	0.3314	0.326
	FASN	1.000	2.351	0.742	1.498	0.6137	0.357
	ACACA	1.000	1.112	0.414	0.840	0.2648	0.339
	GLUT4	1.000	0.415	0.696	0.556	0.2166	0.479
Lipolysis	*ATGL*	1.000[a]	0.441[ab]	0.254[b]	0.363[b]	0.2881	0.006
	HSL	1.000	0.629	0.437	0.537	0.2130	0.389
	LPL	1.000	0.599	0.398	0.301	0.2681	0.302
Adipogenesis	*PPARG*	1.000	1.166	0.845	0.188	0.5761	0.315
	ZFP423	1.000	2.377	1.609	0.470	0.4170	0.290
	CEBPA	1.000	1.053	2.271	1.188	1.1324	0.399
	CEBPB	1.000	0.912	1.455	0.398	0.6778	0.207
Angiogenesis	*VEGFA*	1.000	1.196	0.519	0.840	0.5464	0.411
	VEGFR1	1.000	1.407	0.394	0.444	1.1104	0.592
	VEGFR2	1.000	0.382	0.434	0.277	0.4251	0.067
Subcutaneous (back) fat							
Lipogenesis	*DGAT1*	1.000	0.467	0.307	0.309	0.2842	0.317
	DGAT2	1.000	2.065	1.331	0.806	0.4797	0.511
	FASN	1.000	0.454	0.126	0.549	0.3123	0.123
	ACACA	1.000	0.992	0.447	0.749	0.2259	0.579
	GLUT4	1.000	0.787	1.638	0.948	0.3244	0.502
Lipolysis	*ATGL*	1.000	0.890	0.728	0.222	0.2977	0.172
	HSL	1.000	5.396	2.234	0.345	1.9421	0.094
	LPL	1.000	0.550	0.396	0.463	0.2359	0.163
Adipogenesis	*PPARG*	1.000[a]	1.166[a]	0.845[a]	0.188[b]	0.5146	0.027
	ZFP423	1.000	2.377	1.609	0.470	1.2698	0.207
	CEBPA	1.000[b]	1.723[ab]	2.140[a]	0.347[c]	0.9599	0.031
	CEBPB	1.000	1.943	2.019	0.418	1.1118	0.133
Angiogenesis	*VEGFA*	1.000	4.685	2.521	1.199	2.7824	0.254
	VEGFR1	1.000[a]	1.687[a]	1.745[a]	0.300[b]	0.7997	0.021
	VEGFR2	1.000	1.101	0.845	0.148	0.7626	0.334

ZFP423 = zinc finger protein 423; *PPARG* = peroxisome proliferator activated receptor gamma; *CEBPA* = CCAAT enhancer binding protein alpha; *CEBPB* = CCAAT enhancer binding protein beta; *VEGFA* = vascular endothelial growth factor A; *VEGFR1* = vascular endothelial growth factor receptor 1; *VEGFR2* = vascular endothelial growth factor receptor 2; *LPL* = lipoprotein lipase; *GLUT4* = facilitated glucose transporter 4; *FASN* = fatty acid synthase; *ACACA* = acetyl-CoA carboxylase alpha; *HSL* = hormone-sensitive lipase; *ATGL* = adipose triglyceride lipase; *DGAT1* = diacylglycerol O-acyltransferase 1; *DGAT2* = diacylglycerol O-acyltransferase 2.
[a-c] Means with different letters are significantly different from each other ($P < 0.05$).
[1] The sheep were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU, vitamin A palmitate (VA) with corn oil as solvent, 7500 IU, all-trans-retinoic acid (RA) with corn oil as solvent, and a mixture of 7500 IU, VA-5 mg SU5416 (an inhibitor of the soluble vascular endothelial growth factor receptor) with corn oil as solvent. Data were shown as mean with SEM provided, $n = 6$ in each group.

angiogenesis. It would be worthwhile to further investigate whether other angiogenic nutrients could also increase IMF. Notably, an increase in vasculature not only contributes to adipose tissue development but also enhances muscle growth (Wang et al., 2018). Our upcoming paper will present data on how VA enhances muscle growth via angiogenesis.

While the primary concern revolves around increasing IMF, there is also interest in whether neonatal VA injection influences the overall fatness of sheep. In a previous study involving cattle, neonatal VA injection elevated IMF levels without affecting overall fatness (Yu et al., 2022). However, in the current study, although not statistically significant, VA injection increased the perirenal and omentum fat, likely attributable to the animals' higher body weight. In fact, the rise in fatness stemmed from enhanced body maturation rather than direct effects of VA or RA treatment because the injected retinoids don't stay long in muscle, and this study didn't detect a difference in lipogenic activities in muscles obtained at 8 months of age. It's noteworthy that RA-treated animals also exhibited slightly increased visceral fat weight alongside higher body weight (though not statistically significant), yet intramuscular fat remained unchanged. The distinct impacts of VA and RA on intramuscular fat development warrant further investigation.

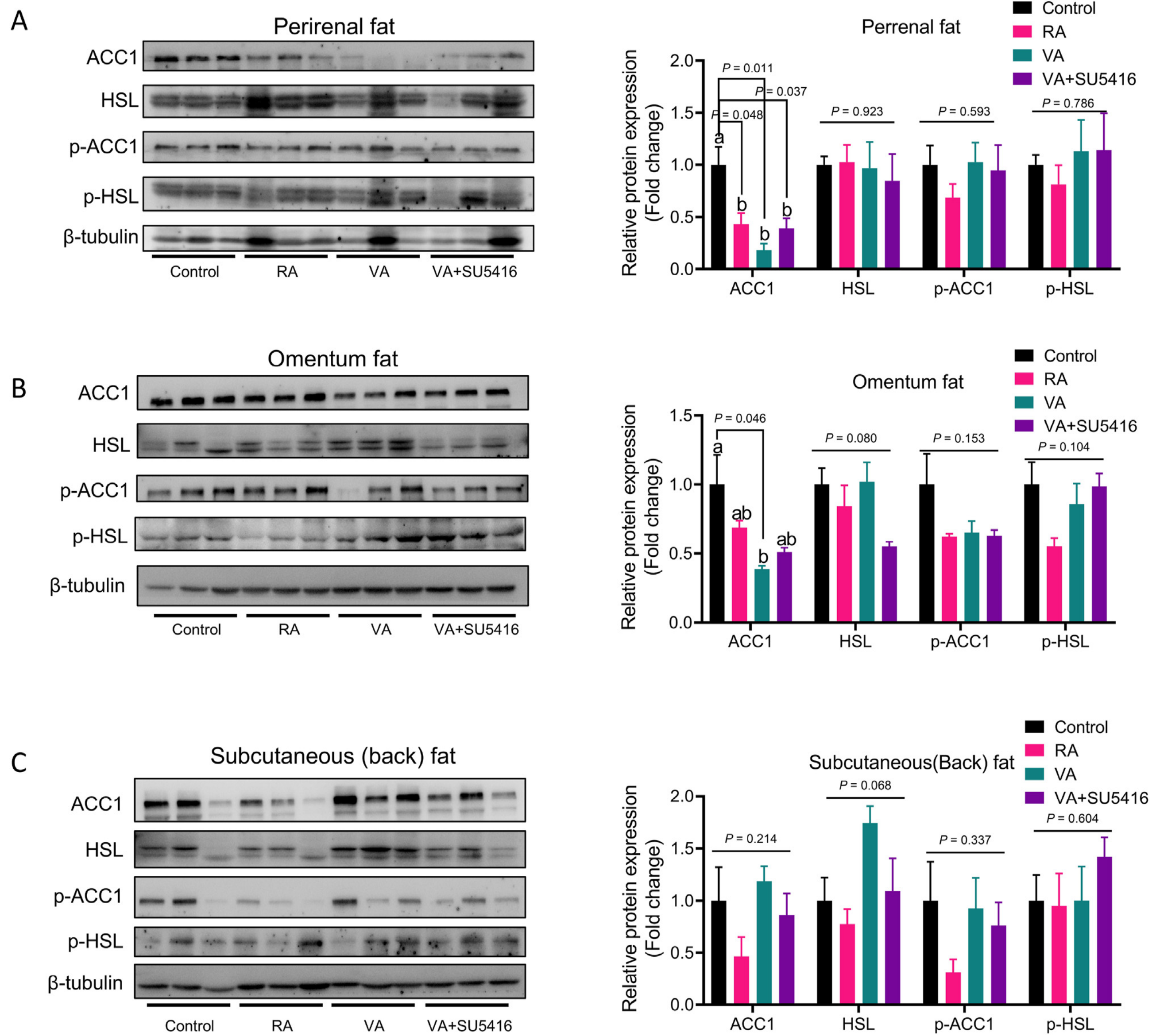

Fig. 2. Neonatal vitamin A injections affect lipogenic and lipolytic protein levels in omentum, perirenal, and subcutaneous (back) fat of 15-day-old sheep. (A) Protein expression of ACC1, HSL, p-ACC1, and p-HSL in perirenal fat of 15-day-old sheep. (B) Protein expression of ACC1, HSL, p-ACC1, and p-HSL in omentum fat of 15-day-old sheep. (C) Protein expression of ACC1, HSL, p-ACC1, and p-HSL in subcutaneous (back) fat of 15-day-old sheep. The sheep were injected intramuscularly into the biceps femoris muscle at 1, 7, 14, and 21 days of age with corn oil (control), 7500 IU vitamin A palmitate (VA) with corn oil as solvent, 7500 IU all-trans-retinoic acid (RA) with corn oil as solvent, and a mixture of 7500 IU VA-5 mg SU5416 (an inhibitor of the soluble vascular endothelial growth factor receptor) with corn oil as solvent. ACC1 = acetyl-CoA carboxylase alpha; HSL = hormone-sensitive lipase; p-ACC1 = phospho-acetyl-CoA carboxylase alpha; p-HSL = phospho-acetyl-hormone-sensitive lipase. Data were shown as mean ± SEM, n = 3 in each group. [a, b] Mean with different letters are significantly different from each other ($P < 0.05$).

5. Conclusion

In conclusion, intramuscular injections of VA but not RA at the neonatal stage improved the growth performance of Hu sheep, increasing the number of intramuscular adipocytes and marbling by promoting angiogenesis.

Credit author contribution statement

Zhongzuo Huang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaoxiao Yu:** Investigation, Formal analysis, Data curation. **Zongyou Jiang:** Investigation. **Gaojian Tang:** Investigation. **Shaoqi Gao:** Investigation. **Yifan Xiang:** Investigation. **Yicheng Luo:** Investigation. **Boping Ye:** Investigation. **Yating Li:** Investigation. **Pengkang Song:** Investigation. **Yu Xin:** Investigation. **Min Du:** Writing – review & editing, Supervision. **Junxing Zhao:** Writing – review & editing, Supervision, Resources, Project administration. **Bo Wang:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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 A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.08.006.

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