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ORIGINAL ARTICLE

Maternal 25-hydroxycholecalciferol during lactation improves intestinal calcium absorption and bone properties in sow-suckling piglet pairs

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Abstract

Lower maternal vitamin D status during lactation is a common health problem. The objectives of this study were to investigate the effects of maternal 25-hydroxycholecalciferol (25-OH-D_3) supplementation during lactation on maternal and neonatal bone health in a sow model. 32 Large White × Landrace sows were assigned randomly to one of two diets supplemented with 2000 IU/kg vitamin D_3 (ND) or 50 μg/kg 25-OH-D_3 (25-D). The experiment began on day 107 of gestation and continued until weaning on day 21 of lactation. Maternal 25-OH-D_3 supplementation significantly decreased milk n-6:n-3 PUFA ratio, which supported bone formation of piglets. Supplementation with 25-OH-D_3 altered bone turnover rate of sows and piglets, as evidenced by higher bone-specific alkaline phosphatase (BALP) concentration in serum. 25-D sows had significantly higher bone density and mechanical properties of tibias and femurs than ND sows. Calcium (Ca) absorption rate was higher in 25-D sows than ND sows, which was caused partially by the increased mRNA expressions of renal 1α-hydroxylase (CYP27B1) and duodenal vitamin D receptor (VDR), transient receptor potential vanilloid 6 (TRPV6), and calcium-binding protein D9k (CaBP-D9k). Maternal 25-OH-D_3 supplementation increased tibial and femoral Ca content by up-regulating Ca-related gene expression in kidney (CYP27B1), ileum (VDR and claudin-2), and colon (VDR and CaBP-D9k), thus, activating 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3]-dependent Ca transport in piglets. In conclusion, improved milk fatty acids and higher mRNA expressions of calcitropic genes triggered by maternal 25-OH-D_3 supplementation would be the potential mechanism underlying the positive effects of 25-OH-D_3 on maternal and neonatal bone health.

Keywords Maternal 25-hydroxycholecalciferol · Milk fatty acids · Calcium absorption · Bone · Piglets

Introduction

Calcium (Ca) is the primary mineral in animals that plays important roles in multifarious physiological processes such as the formation of bone structure, muscle contraction, excitability and permeability of plasma membrane, neurotransmission, and signal transduction pathways [1, 2]. Lactation period represents a challenge for the mother to provide the necessary Ca for the developing neonate [3]. The physiological requirement of Ca is markedly elevated as a result of fetal growth and milk production [4]. The demand for Ca may be fulfilled by enhanced intestinal Ca absorption, increased renal Ca reabsorption, and mobilization and loss of Ca from the maternal skeleton [5]. Breastfeeding is associated with increased bone turnover, which results in the release of Ca from the maternal skeleton during lactation [6]. However, intervention measures limited to Ca supplementation only had a slight effect on bone loss during lactation [7].

It is accepted that vitamin D plays an important role in the maintenance of optimal Ca homeostasis for bone mineralization and skeletal muscle development. 25-hydroxycholecalciferol (25-OH-D_3) is the hydroxylated metabolite of vitamin D_3 in the liver. And 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] is produced mainly, but not exclusively, in the kidney via 1α-hydroxylase (CYP27B1) [8]. Circulating 25-OH-D_3 is considered as the major indicator of vitamin

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D status in blood [9]. 25-OH-D_3 is transported from the mother to the fetus through the placenta during pregnancy, and the concentration of 25-OH-D_3 in umbilical cord blood is about 80% of that in maternal blood at birth [10]. However, only minimal amounts of maternal blood 25-OH-D_3 could be transferred to maternal breast milk [11]. Therefore, maternal vitamin D status must be much higher during lactation in comparison with pregnancy, which is critical for the long-term health of the dyad. Vitamin D receptor (VDR) is present in mammary gland [12], so vitamin D may improve milk composition and further influence growth and health of progeny. Vitamin D deficiency has adverse influences on the skeletal system and may be a potential risk factor for various diseases including cancer, cardiovascular and metabolic disease, diabetes, and autoimmune diseases [13]. Maternal vitamin D insufficiency is a risk marker for reduced fertility and various adverse outcomes of pregnancy [11]. It has been associated with lower vitamin D content of breast milk, lower birth weight, and lower bone mineral content of the offspring at birth [10]. Vitamin D supplementation during lactation may ensure appropriate maternal vitamin D status in cases of lower vitamin D dietary intake and restricted synthesis in the skin.

Cashman et al. [14] suggested that 25-OH-D_3 has a greater absorption efficiency compared with regular vitamin D_3. However, there were few studies about the relationship between maternal 25-OH-D_3 supplementation during lactation and bone properties of maternal and neonatal pairs. In addition, potential regulatory effects of maternal vitamin D status during lactation on intestinal Ca absorption of progeny have not been clearly studied. Given this background, the current research was to investigate the effects of maternal 25-OH-D_3 supplementation during lactation on maternal and neonatal bone properties, as well as gene expressions related to intestinal Ca absorption of neonate in a lactating sow model. It is supposed to provide some mechanistic insights into the application of 25-OH-D_3 to lactation diets for improving maternal and neonatal health.

Materials and methods

Animal, diets, and experimental design

32 Large White × Landrace sows (body weight 262.30 ± 3.61 kg; parity 3.81 ± 0.26) were used in this study. Sows were weighed on day 107 of gestation and allotted randomly to two treatments based on their body weight, parity, and backfat. The diet of ND treatment was supplemented with 2000 IU/kg vitamin D_3 (equivalent to 50 μg/kg feed), and the diet of 25-D treatment was supplemented with 50 μg/kg 25-OH-D_3 (Haineng 25-D). 25-OH-D_3 was obtained from Haineng Bioengineering Co., Ltd. (Rizhao, China). Other nutrients met or exceeded nutrient requirements as recommended by the National Research Council (NRC) [15]. The composition and nutrient levels of the diet are shown in Supplementary Table 1.

The experiment started on day 107 of gestation when sows were moved to the individual farrowing crates until their piglets were weaned on day 21 of lactation. The farrowing room was strictly controlled, and the room temperature was always maintained at a minimum of 20 °C, while supplemental heat was provided to piglets using heat lamps. Lights in the farrowing room were on from 06:00 to 16:00 h. Sows were fed 2.0 kg/day experimental diets from day 107 of gestation to farrowing and then allowed ad libitum access to experimental diets and water during the whole lactating period. Fresh feed was provided at 06:00, 11:00, and 16:00 h every day. Feed intake of each sow was recorded to evaluate average daily feed intake. Sow body weights and backfat depth were recorded on day 107 of gestation, at farrowing and at weaning. The backfat thickness was measured at the P2 position (6 cm from the midline at the head of the last rib) [16]. Piglets were treated according to the routine management practices that included teeth clipping, tail docking, ear notching, and subcutaneous iron dextran injections (200 mg/pig) within 24 h postpartum. Body weight of piglets was recorded at birth and weaning to calculate average daily weight gain of piglets.

Sample collection

Colostrum samples were collected by hand milking from all functional mammary glands of each sow within 2 h after parturition, and milk samples were collected after intramuscular injection with 2 mL oxytocin on days 7, 14, and 21 of lactation. Sows were given feed at 06:00 h, allowed 1 h to consume to satiety, and residual feed was removed. The blood samples were collected from the ear marginal vein at 10:00 h using 5 mL vacuum tubes (Becton–Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). After farrowing, umbilical cord blood samples were collected from as many umbilical cords as possible. On days 0, 7, 14, and 21 of lactation, blood samples of piglets were collected from jugular vein using vacuum tubes (Becton–Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Blood samples were centrifuged at 3000×*g* at 4 °C for 10 min. All samples were stored at − 80 °C until further analysis. From days 19 to 21 of lactation, feces samples (200 g) were collected from lactating sows and stored at − 20 °C for Ca and P absorption analysis.

At weaning, four sows and six piglets (one piglet close to the average body weight of each litter) per treatment were euthanized humanely. Cortex of the left kidney and segments of the mid-ileum and mid-colon were collected and quickly placed in RNAase-free tubes, frozen in liquid nitrogen, and

then stored at − 80 °C for further analysis. Tibias and femurs from the hind leg of each pig were excised and freed of surrounding soft tissues. Bones were frozen at − 20 °C until bone analysis.

Analysis of colostrum and milk samples

To determine 25-OH-D_3 concentration, colostrum and milk samples should been prepared with adequate extraction. As described by Gomes et al. [17], protein precipitation was performed with acetonitrile at room temperature. Hexane:dichloromethane (4:1, v/v) solution was added to the supernatants prepared in protein precipitation. The upper organic layers were evaporated to dryness under nitrogen at room temperature. The residues from colostrum and milk samples were analyzed using liquid chromatography–tandem mass spectrometry system (LC–MS/MS 6430, Agilent Technologies, USA). Fatty acid compositions were analyzed using a gas chromatograph (6890 series, Agilent Technologies, Wilmington, DE). Lipid samples were converted to fatty acid methyl esters using methanolic HCl solution. Undecanoic acid (C11:0) was used as the internal standard. Fatty acids were expressed as the proportion of each individual fatty acid to the total of all fatty acid present in the milk samples.

Serum measurement

Serum 25-OH-D_3 and 1,25-$(OH)_2$-D_3 were determined using liquid chromatography–tandem mass spectrometry system (LC–MS/MS 6430, Agilent Technologies, USA). Serum Ca and phosphorus (P) concentrations were determined by colorimetry using commercial kits (Zhongsheng Beikong Biotechnology & Science Inc., Beijing, China). Serum bone-specific alkaline phosphatase (BALP) and tartrate-resistant acid phosphatase (TRAP) were analyzed using commercial kits (Zhongsheng Beikong Bio-technology & Science Inc., Beijing, China). Serum concentrations of osteocalcin (OC), parathyroid hormone (PTH), and insulin-like growth factor-I (IGF-I) were assessed using commercially available ELISA test kit (Sino-UK institute of Biological Technology, Beijing, China).

Ca and P absorption and fecal excretion

Ca and P absorption rates were determined by indicator method [18]. Fecal samples were dried in an oven (65 °C) for 72 h and milled. Diet and fecal samples were analyzed for Ca and P contents according to the Association of Official Analytical Chemists (AOAC) [19]. Chromium (Cr) content was analyzed using an atomic absorption spectrophotometer (Z-5000 Automatic Absorption Spectrophotometer; Hitachi, Tokyo, Japan). The equations for apparent absorption: absorption rate $(\%) = 1 - (Cr_{diet} \times M_{feces})/(Cr_{feces} \times M_{diet})$, where Cr is the amount of chromic oxide in the diets or feces and M is the amount of Ca or P in the diets or feces.

Bone density and biomechanical testing

Tibias and femurs were used to determine bone density and mechanical properties. Total bone density was calculated according to the Archimedes principle [20]. In brief, bone samples were weighed submerged in distilled water and then weighed out of water. Total density was calculated using the formula: density $= (A/A - B) \times P$, where A is the weight of the hydrated bone out of water, B is the weight of the hydrated bone submerged in water, $A - B$ is the difference in weight, and P is the density of distilled water. Three-point bending test was carried out using MTS Material Testing Apparatus (Model 810, MTS Systems Corporation, Minneapolis, USA) to analyze the biomechanical properties of bones. Load was applied to the midpoint of the bone, which was held by two supports spaced 30 mm apart. Load–displacement curves were recorded at a compression speed of 10 mm/min, and breaking force (N), failure deflection (mm), stiffness (N/mm), and absorbed energy (J) were measured.

Determination of bone chemical composition

For piglets, the left tibias and femurs were used to determine ash, Ca and P contents. Contents of ash, Ca and P were determined on a fat-free basis as previously described [21]. Bones were wrapped with gauze, boiled in deionized water for 2 h, dried at 55 °C for 24 h, and extracted with ethyl ether for 4 days. After the fat-free dry weight was obtained, bones were placed in a muffle furnace at 600 °C for 16 h. Ashes were used for Ca and P analysis. For sows, mid-diaphysis of tibias and femurs was used to determine bone ash, Ca and P contents.

The right tibias and femurs of piglets were separated from periosteum and marrow, cooled in liquid nitrogen, and pulverized for lipid extraction. Fatty acid compositions were analyzed using a gas chromatograph (HP 6890 Series GC system). Prostaglandin E_2 (PGE_2) content of tibias and femurs were detected using commercially available ELISA test kit (Sino-UK Institute of Biological Technology, Beijing, China).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from kidney, duodenum, ileum, and colon using Trizol reagent (CWBIO Biotech Co., Beijing, China). Reverse transcription PCR was conducted using the PrimeScript^{RT} Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The mRNA expressions of CYP27B1, 24-hydroxylase (CYP24A1),

VDR, transient receptor potential vanilloid 5 (TRPV5), calcium-binding protein D28 k (CaBP-D28 k), sodium-phosphate cotransporter 1 (NaPi-IIa) in kidney, and CYP24A1, VDR, claudin-2, transient receptor potential vanilloid 6 (TRPV6), calcium-binding protein D9k (CaBP-D9k), and sodium-phosphate cotransporter 2 (NaPi-IIb) in duodenum, ileum, and colon were measured by real-time quantitative PCR with SYBR Green (TaKaRa) using the ABI 7500 real-time PCR system (Life Technologies, USA). All samples were analyzed in duplicate, and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of each target gene [22]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The following sequences of primers were used: CaBP-D9k forward: GCTTCAGAC GGAATTCCCCA, reverse: TCCATCACCGTTCTTATC CAGT; CaBP-D28k forward: TGAGCTTTTGCTCACTCC CC, reverse: ACTTCCGTCAGCGTCGAAAT; Claudin-2 forward: TTGGCCTGTATCATCACCGT, reverse: ATG AAGATTCCACGCAACGG; CYP24A1 forward: GCA AGAATGAAGCTTTTGGC, reverse: AGACACCAAGGT CAACCAGG; CYP27B1 forward: AATGACCATAGCAAA GTACTTGAGG, reverse: GTCAGTAGTTGAATTCTTCCT CAGC; GAPDH forward: TCGGAGTGAACGGATTTGGC, reverse: TGACAAGCTTCCCGTTCTCC; NaPi-IIa forward: ACTCGACTTGTGGTAGCCTC, reverse: AGTGAAAGG CTCCGTGATGA; NaPi-IIb forward: ACCGTTCTCCAC CAAATTGC, reverse: AGGGGACGGTGACATTCTTT; TRPV5 forward: CTTCGCCTTCGCCATCATTG, reverse: GCAGCGCATTCTGATCTTGG; TRPV6 forward: CAC TGGGTGTCCCAAAGTCC, reverse: ACTGGCCAGACA CAGAGACT; VDR forward: AGGCTTCTTCAGACGGAG CATGAA, reverse: ACTCCTTCATCATGCCGATGTCCA.

Statistical analysis

All data were performed using SAS 9.4 (SAS Inst. Inc., Cary, NC). Each sow (litter) or piglet was considered as the experimental unit. Models included treatment as the fixed effect and replicate as the random effect. The independent sample *t* test procedure of SAS was performed to detect differences between ND and 25-D treatments. Milk fatty acids, serum, and milk 25-OH-D_3 were analyzed using two-factor repeated measurements of SAS (maternal vitamin D type with collection time point as the repeated measurement). Interactive effects were defined as an interaction between maternal vitamin D type and day of lactation. Correlations between changes of serum and milk 25-OH-D_3 concentrations were tested by Pearson's correlation analysis of SAS system. Significant differences were declared at $P < 0.05$ between ND and 25-D treatments. Data were presented as means of each treatment and standard errors of the means. Differences between $0.05 \le P < 0.10$ were considered as a trend toward significance.

Results

Reproductive performance

Maternal 25-OH-D_3 supplementation increased ($P < 0.05$) total litter weight gain compared with ND treatment (Supplementary Table 2). Piglets from 25-D treatment tended to have higher ($P = 0.08$) average daily gain than piglets suckling ND sows.

Fatty acid composition of colostrum and milk

As shown in Table 1, maternal 25-OH-D_3 supplementation significantly decreased ($P < 0.05$) milk n-6:n-3 PUFA ratio. In addition, milk C20:4n-6 content tended to be lower ($P = 0.05$) due to maternal 25-OH-D_3 supplementation. The main effect of lactation day was observed for all identified fatty acids except milk C18:1n-9, C20:1 and n-6:n-3 PUFA ratio. An interactive effect of maternal vitamin D type and day of lactation was observed for milk C16:0 ($P = 0.01$), C18:1n-9 ($P = 0.04$), SFA ($P = 0.02$), and MUFA ($P = 0.02$).

Changes in serum and milk 25-OH-D_3 levels and their correlations

At farrowing and weaning, an interactive effect of maternal vitamin D type and day of lactation on serum 25-OH-D_3 is summarized in Fig. 1a. Analysis of serum vitamin D status showed interactive effects for serum 25-OH-D_3 concentration in sows ($P < 0.001$) and piglets ($P < 0.001$). The main effect of vitamin D type or day of lactation was also observed for serum 25-OH-D_3 ($P < 0.001$). Maternal 25-D treatment increased ($P < 0.05$) serum 25-OH-D_3 concentration in the umbilical cords, which led to higher serum 25-OH-D_3 concentration of piglets born from 25-D sows. An interactive effect of maternal vitamin D type and day of lactation was observed for milk 25-OH-D_3 ($P < 0.001$) (Fig. 1b). No significant difference was found in colostrum 25-OH-D_3 level between ND and 25-D treatments. However, 25-OH-D_3 concentration of milk was higher ($P < 0.05$) in 25-D sows compared with ND sows on days 7, 14, and 21 of lactation. A main effect of lactation day was also observed for milk 25-OH-D_3 ($P < 0.001$). Correlation analysis showed that change of serum 25-OH-D_3 concentration was correlated to change of milk 25-OH-D_3 concentration ($R = 0.82$–0.86, $P < 0.01$) (Fig. 1c).

Mineral absorption and their fecal excretion

As shown in Fig. 2, Ca absorption rate of 25-D sows was higher ($P < 0.05$) than that of ND sows and its fecal excretion

Table 1 Effects of dietary vitamin D_3 source on milk fatty acid composition of sows at farrowing and weaning (g/100 g total fatty acids)

Items	ND		25-D		*P* value		
	Day 0	Day 21	Day 0	Day 21	Vitamin D type	Day	Vitamin D type × day
C10:0	0.043 ± 0.004	0.269 ± 0.015	0.053 ± 0.007	0.281 ± 0.030	0.58	< 0.001	0.93
C12:0	0.055 ± 0.007	0.301 ± 0.012	0.057 ± 0.006	0.304 ± 0.011	0.79	< 0.001	0.95
C14:0	1.73 ± 0.13	3.64 ± 0.11	1.55 ± 0.10	3.61 ± 0.11	0.33	< 0.001	0.53
C14:1	0.027 ± 0.006	0.259 ± 0.022	0.030 ± 0.004	0.208 ± 0.016	0.14	< 0.001	0.07
C15:0	0.142 ± 0.005	0.089 ± 0.003	0.132 ± 0.007	0.095 ± 0.011	0.81	< 0.001	0.15
C16:0	21.02 ± 0.96	31.79 ± 0.62	19.97 ± 0.38	34.23 ± 0.43	0.35	< 0.001	0.01
C16:1	1.94 ± 0.25	9.51 ± 0.65	2.30 ± 0.21	8.65 ± 0.15	0.55	< 0.001	0.09
C17:0	0.267 ± 0.017	0.145 ± 0.006	0.257 ± 0.009	0.173 ± 0.019	0.58	< 0.001	0.16
C18:0	4.77 ± 0.27	3.83 ± 0.17	4.90 ± 0.25	4.17 ± 0.19	0.45	< 0.001	0.38
C18:1n-9	24.09 ± 0.99	25.21 ± 1.09	27.81 ± 1.63	24.55 ± 0.99	0.31	0.28	0.04
C18:2n-6	38.02 ± 2.25	20.74 ± 0.42	35.41 ± 1.62	19.62 ± 0.72	0.27	< 0.001	0.57
C18:3n-3	3.04 ± 0.30	1.74 ± 0.06	3.33 ± 0.07	1.93 ± 0.07	0.20	< 0.001	0.76
C20:0	0.145 ± 0.009	0.110 ± 0.006	0.134 ± 0.005	0.113 ± 0.002	0.63	< 0.001	0.11
C20:1	0.203 ± 0.013	0.229 ± 0.028	0.229 ± 0.022	0.247 ± 0.043	0.56	0.27	0.85
C20:3n-3	0.166 ± 0.010	0.063 ± 0.006	0.151 ± 0.007	0.070 ± 0.014	0.72	< 0.001	0.22
C20:3n-6	0.351 ± 0.047	0.077 ± 0.007	0.291 ± 0.020	0.078 ± 0.007	0.30	< 0.001	0.25
C20:4n-6	1.24 ± 0.07	0.446 ± 0.007	1.13 ± 0.07	0.366 ± 0.014	0.05	< 0.001	0.80
C20:5n-3	0.134 ± 0.015	0.039 ± 0.004	0.113 ± 0.008	0.043 ± 0.004	0.40	< 0.001	0.15
C21:0	0.648 ± 0.027	0.310 ± 0.037	0.587 ± 0.017	0.318 ± 0.049	0.48	< 0.001	0.31
C22:0	0.092 ± 0.006	0.076 ± 0.003	0.085 ± 0.008	0.072 ± 0.002	0.31	0.01	0.77
C22:1n-9	0.357 ± 0.020	0.196 ± 0.008	0.436 ± 0.062	0.190 ± 0.008	0.27	< 0.001	0.24
C22:6n-3	0.260 ± 0.026	0.039 ± 0.002	0.211 ± 0.009	0.041 ± 0.006	0.12	< 0.001	0.11
SFA	28.27 ± 1.32	40.25 ± 0.58	27.13 ± 0.41	43.04 ± 0.48	0.35	< 0.001	0.02
MUFA	26.62 ± 1.24	35.41 ± 0.81	30.81 ± 1.78	33.84 ± 1.09	0.39	< 0.001	0.02
PUFA	43.21 ± 2.54	23.15 ± 0.45	40.63 ± 1.76	22.18 ± 0.82	0.34	< 0.001	0.58
n-6 PUFA	39.61 ± 2.25	21.26 ± 0.46	36.83 ± 1.69	20.10 ± 0.77	0.25	< 0.001	0.55
n-3 PUFA	3.60 ± 0.31	1.88 ± 0.05	3.80 ± 0.07	2.07 ± 0.06	0.27	< 0.001	0.98
n-6:n-3	11.19 ± 0.57	11.34 ± 0.44	9.67 ± 0.32	9.69 ± 0.19	< 0.001	0.86	0.89

Values were given as mean ± SEM ($n = 6$)

SAT = C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0

MUFA = C14:1 + C16:1 + C18:1 + C20:1 + C22:1

PUFA = C18:2 + C18:3 + C20:3 + C20:4 + C20:5 + C22:6

n-6:n-3 = (C18:2 + C20:3 + C20:4)/(C18:3 + C20:5 + C22:6)

was significantly lower than that of ND sows. P absorption rate and its fecal excretion did not differ between ND and 25-D treatments.

Serum Ca, P, and bone turnover markers of sows and piglets

At weaning, P concentration of sow serum tended to be increased ($P = 0.08$) with 25-OH-D_3 supplementation (Table 2), and significant increases of serum BALP and IGF-I concentrations were found in 25-D sows. At farrowing and weaning, 25-D sows had higher ($P < 0.05$) serum 1,25-$(OH)_2$-D_3 concentration compared with ND sows. On day 21 of lactation, piglets suckling 25-D sows had higher ($P < 0.05$) serum BALP and IGF-I concentrations compared with piglets suckling ND sows (Table 3). Significant increase of serum 1,25-$(OH)_2$-D_3 concentration was found in piglets suckling 25-D sows on days 14 and 21 of lactation.

Bone properties of sows

25-D sows had higher ($P < 0.05$) Ca content and total bone density than ND sows in tibias and femurs (Table 4). Three-point bending tests at the mid-diaphysis of tibias and femurs demonstrated that 25-D sows had higher ($P < 0.05$) breaking force and stiffness compared with ND sows.

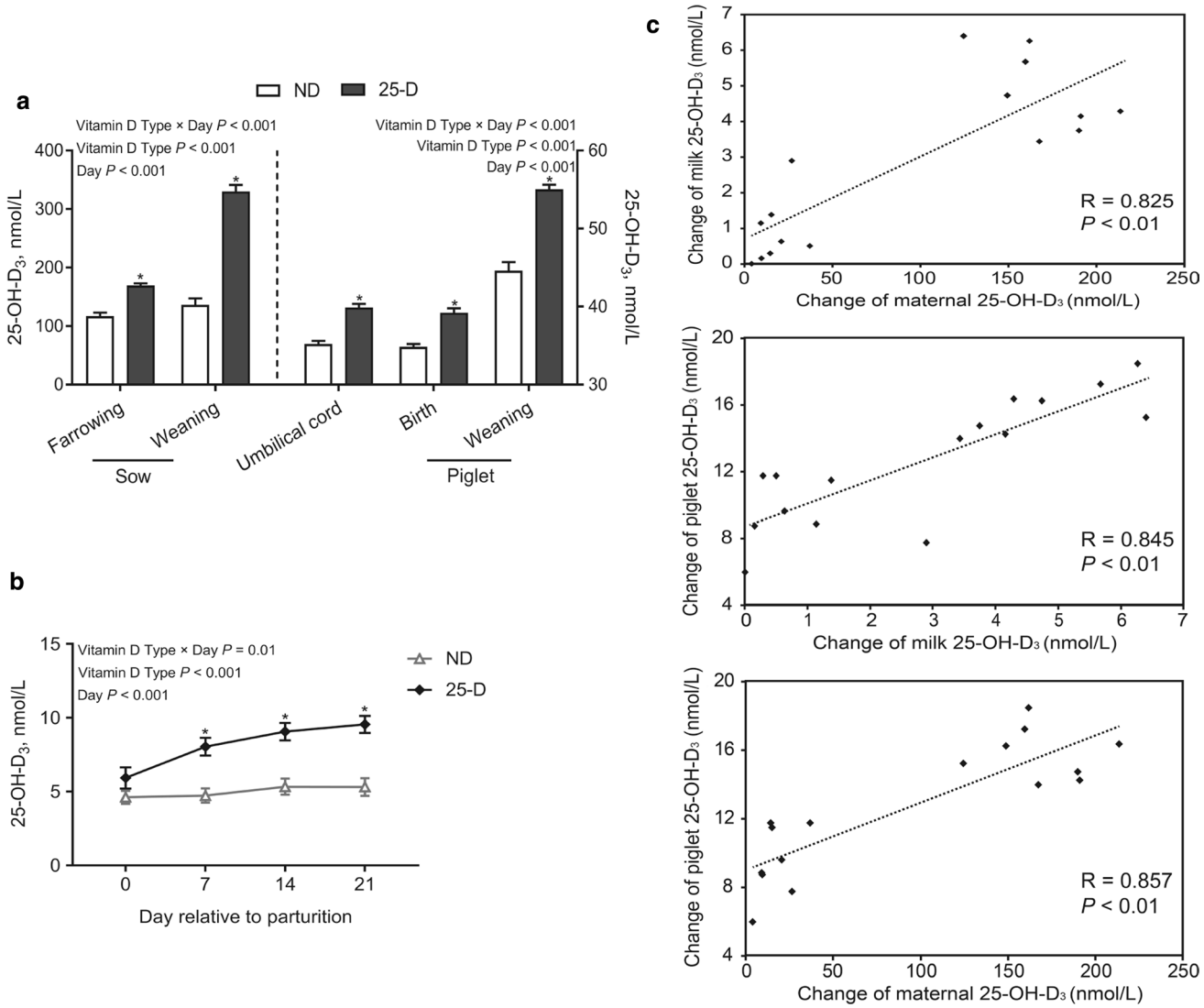

Fig. 1 Changes in serum and milk 25-OH-D_3 levels and their correlations during lactation. **a** 25-OH-D_3 levels in serum of sow, umbilical cord, and piglet. **b** 25-OH-D_3 levels in colostrum and milk. **c** The correlations of changes in serum and milk 25-OH-D_3 levels. Values were given as mean ± SEM ($n = 8$).*$P < 0.05$ versus ND treatment within the same sampling day

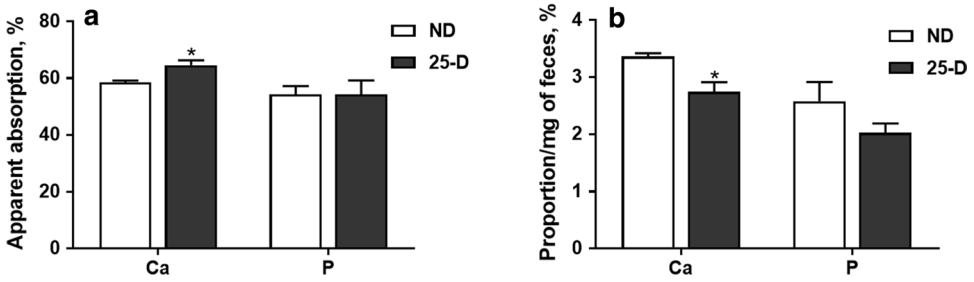

Fig. 2 Effects of dietary vitamin D_3 source on Ca and P absorption and their fecal excretion. **a** Apparent absorption of Ca and P. **b** Percentage contents of Ca and P in feces. Values were given as means ± SEM ($n = 4$). *$P < 0.05$ versus ND treatment

Bone fatty acid composition

As shown in Table 5, a significant decrease of n-6:n-3 PUFA ratio in tibias was found in piglets suckling 25-D sows. In femurs, a significant decrease of C20:4n-6 content was noticed in piglets suckling 25-D sows, and n-6:n-3 PUFA ratio tended to be higher ($P = 0.06$) in piglets suckling 25-D sows compared with piglets from ND treatment.

Table 2 Effects of dietary vitamin D_3 source on bone biochemical markers in sow serum

Items	ND	25-D	*P* value
Farrowing			
Ca, mmol/L	2.26 ± 0.03	2.32 ± 0.03	0.21
P, mmol/L	2.37 ± 0.06	2.40 ± 0.07	0.74
BALP, U/L	18.58 ± 3.09	19.40 ± 1.87	0.83
TRAP, U/L	4.80 ± 0.22	5.04 ± 0.13	0.64
OC, ng/mL	5.06 ± 0.37	5.32 ± 0.48	0.68
PTH, pg/mL	64.00 ± 1.61	64.25 ± 1.24	0.90
IGF-I, ng/mL	223 ± 25	237 ± 18	0.66
1,25-$(OH)_2$-D_3, pmol/L	129 ± 6	164 ± 13	0.03
Weaning			
Ca, mmol/L	2.35 ± 0.05	2.36 ± 0.06	0.94
P, mmol/L	1.47 ± 0.19	2.17 ± 0.32	0.08
BALP, U/L	33.45 ± 2.97	54.91 ± 2.78	< 0.01
TRAP, U/L	5.25 ± 0.44	5.23 ± 0.29	0.99
OC, ng/mL	5.80 ± 0.47	5.75 ± 0.44	0.94
PTH, pg/mL	63.42 ± 2.96	63.03 ± 1.28	0.91
IGF-I, ng/mL	245 ± 2	266 ± 8	0.02
1,25-$(OH)_2$-D_3, pmol/L	120 ± 7	249 ± 15	< 0.01

Values were given as mean ± SEM, $n = 8$ sows for data on Ca, P, BALP, and TRAP; and $n = 6$ sows for data on PTH and IGF-I

Table 3 Effects of vitamin D_3 source in maternal diets on bone biochemical markers in piglet serum

Items	ND	25-D	*P* value
Day 7			
Ca, mmol/L	2.74 ± 0.06	2.84 ± 0.08	0.34
P, mmol/L	2.98 ± 0.07	3.00 ± 0.10	0.88
BALP, U/L	470 ± 41	517 ± 66	0.56
TRAP, U/L	37.55 ± 3.18	23.72 ± 1.23	0.07
OC, ng/mL	6.86 ± 0.27	6.50 ± 0.48	0.52
PTH, pg/mL	124.70 ± 13.96	119.99 ± 7.91	0.78
IGF-I, ng/mL	102.21 ± 2.73	106.92 ± 1.13	0.16
1,25-$(OH)_2$-D_3, pmol/L	46.10 ± 1.44	48.92 ± 0.72	0.11
Day 14			
Ca, mmol/L	2.66 ± 0.07	2.84 ± 0.07	0.08
P, mmol/L	3.01 ± 0.10	3.12 ± 0.06	0.40
BALP, U/L	279 ± 12	288 ± 9	0.57
TRAP, U/L	28.23 ± 1.88	24.73 ± 3.10	0.58
OC, ng/mL	7.19 ± 0.44	7.07 ± 0.45	0.85
PTH, pg/mL	124.69 ± 6.27	119.56 ± 6.84	0.59
IGF-I, ng/mL	102.04 ± 2.70	104.91 ± 2.12	0.42
1,25-$(OH)_2$-D_3, pmol/L	54.19 ± 1.46	61.95 ± 1.90	< 0.01
Day 21			
Ca, mmol/L	2.54 ± 0.06	2.55 ± 0.06	0.96
P, mmol/L	3.13 ± 0.15	3.23 ± 0.16	0.66
BALP, U/L	118 ± 12	197 ± 18	< 0.01
TRAP, U/L	24.65 ± 2.05	16.28 ± 0.72	0.05
OC, ng/mL	4.50 ± 0.43	4.61 ± 0.55	0.87
PTH, pg/mL	142.83 ± 9.73	140.06 ± 11.09	0.85
IGF-I, ng/mL	97.05 ± 2.50	104.71 ± 0.82	0.02
1,25-$(OH)_2$-D_3, pmol/L	59.48 ± 2.24	70.45 ± 4.37	0.04

Values were given as mean ± SEM, $n = 8$ piglets (one piglet per litter) for data on Ca, P, BALP, and TRAP; and $n = 6$ piglets (one piglet per litter) for data on PTH and IGF-I

Bone properties of piglets

As shown in Table 6, piglets suckling 25-D sows had higher ($P < 0.05$) tibial Ca content compared with piglets suckling ND sows, and femoral Ca content tended to be increased ($P = 0.08$) in piglets suckling 25-D sows. Femoral PGE_2 concentration was lower ($P < 0.05$) in piglets suckling 25-D sows compared with piglets from ND treatment. Three-point bending tests at the mid-diaphysis of tibias and femurs demonstrated that piglets suckling 25-D sows had a tendency toward an increased breaking force compared with piglets suckling ND sows.

Gene expression involved in vitamin D_3 metabolism and Ca and P absorption

The mRNA expressions of renal CYP27B1 and CYP24A1, duodenal VDR, TRPV6, and CaBP-D9k were higher ($P < 0.05$) in 25-D sows compared with ND sows (Fig. 3). As shown in Fig. 4a, the expression of renal CYP27B1 in piglets suckling 25-D sows was higher ($P < 0.05$) compared with piglets suckling ND sows. The mRNA expressions of ileal VDR and claudin-2 in piglets suckling 25-D sows were higher ($P < 0.05$) than piglets suckling ND sows (Fig. 4b). Significant increases of colonic VDR and CaBP-D9k mRNA expressions were found in piglets suckling 25-D sows (Fig. 4c). For phosphorus transporters, the mRNA expressions of NaPi-IIa and NaPi-IIb did not differ between ND and 25-D treatments.

Discussion

Although rodents have been extensively considered as animal model for human researches, the discrepancies in physiology and metabolism between humans and rodents are worth considering. The similarities in anatomic, reproductive, and musculoskeletal systems between humans and pigs put the pigs in a better position over other non-primate models [23].

Our present study showed that body weight loss and average daily feed intake of sows during lactation did not differ between ND sows and 25-D sows. This result consisted with those of Lauridsen et al. [24], who reported that maternal 25-OH-D_3 supply did not influence sows'

Table 4 Effects of dietary vitamin D_3 source on bone properties of sows

Items	ND	25-D	*P* value
Tibia			
Weight, g	338 ± 15	324 ± 3	0.39
Ash content, %	45.06 ± 0.54	45.61 ± 0.74	0.58
Ca content, %	15.88 ± 0.28	17.03 ± 0.22	0.02
P content, %	7.94 ± 0.46	7.96 ± 0.63	0.98
Bone density, g/cm^3	1.38 ± 0.01	1.43 ± 0.02	0.02
Breaking force, N	3634 ± 86	3995 ± 95	0.03
Failure deflection, mm	6.03 ± 1.24	6.02 ± 2.01	0.99
Stiffness, N/mm	260 ± 27	788 ± 101	0.01
Absorbed energy, J	23.93 ± 4.77	23.43 ± 5.29	0.95
Femur			
Weight, g	591 ± 57	592 ± 32	0.99
Ash content, %	44.38 ± 0.95	45.90 ± 0.81	0.69
Ca content, %	16.08 ± 0.07	16.88 ± 0.21	0.03
P content, %	7.85 ± 0.47	8.07 ± 0.45	0.74
Bone density, g/cm^3	1.34 ± 0.01	1.41 ± 0.02	0.01
Breaking force, N	5312 ± 306	7519 ± 418	< 0.01
Failure deflection, mm	10.02 ± 1.96	9.74 ± 3.14	0.94
Stiffness, N/mm	424 ± 55	1348 ± 195	0.01
Absorbed energy, J	40.35 ± 8.44	35.78 ± 8.36	0.71

Values were given as mean ± SEM ($n = 4$)

Ash, Ca and P contents were calculated by dividing the ash, Ca and P weights by fat-free dry weight of mid-diaphysis

body weight change and average daily feed intake during lactation. However, maternal 25-OH-D_3 supplementation improved average daily gain of piglets during lactation. 25-OH-D_3 supplementation in maternal diets may influence litter performance through regulation of milk composition or its vitamin D status.

Maternal 25-OH-D_3 could transport to fetus via the umbilical cord [25]. In the present study, 25-OH-D_3 level in umbilical cord blood and neonatal serum from 25-D sows were significantly higher than that form ND sows, which was caused by increased vitamin D status of 25-D sows. Transfer of vitamin D_3 metabolites into breast milk of human was rather limited [26], but milk 25-OH-D_3 level was found to correspond with maternal serum 25-OH-D_3 concentration [27]. As shown in our results of correlation analysis, change of milk 25-OH-D_3 concentration was correlated to change of maternal serum 25-OH-D_3 concentration ($R = 0.825$, $P < 0.01$). 25-OH-D_3 supplementation markedly increased milk 25-OH-D_3 concentration, but 25-OH-D_3 concentration in colostrum did not differ between ND and 25-D treatments. Weber et al. [28] previously reported that 25-OH-D_3 concentration of colostrum was higher than control for first parity sows receiving 25-OH-D_3 supplementation, but 25-OH-D_3 concentration in colostrum of older sows did not differ between 25-OH-D_3-supplemented and unsupplemented sows. Therefore, the parity of sows used may be an important reason for this result in our study.

Dietary n-6:n-3 PUFA ratio has a profound influence on bone fatty acid composition and biosynthesis of prostaglandins, which regulate bone formation and resorption [29]. PGE_2, an eicosanoid derivative of C20:4n-6, is considered a powerful regulating factor for bone modeling and remodeling, and its effect on bone metabolism is dose dependent [30]. Excess production of PGE_2 from higher dietary C20:4n-6 level inhibits bone formation. The previous research suggested that serum BALP activity was greater in rats fed a diet with a low ratio of n-6:n-3 PUFA [31]. In our study, we found that bone fatty acid composition of piglets reflected n-6:n-3 PUFA ratio in sow milk. The C20:4n-6 content and n-6:n-3 PUFA ratio declined in milk, which resulted in significant decreases of tibial n-6:n-3 PUFA ratio and femoral C20:4n-6 and PGE_2 contents and declining tendency of femoral n-6:n-3 PUFA ratio. These data suggested that maternal 25-OH-D_3 supplementation during lactation could promote neonatal bone formation via modulating milk fatty acid composition such as decreased C20:4n-6 content and n-6:n-3 PUFA ratio. This is a novel result, since little information is available about how maternal 25-OH-D_3 supplementation acts on milk fatty acid composition. VDR and 1α-hydroxylase are present in mammary gland [32], and vitamin D supplementation influences milk quality and composition [33]. Recently, Kang et al. [34] reported that vitamin D_3 could regulate the expression of various lipogenic genes including fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), and acetyl-CoA carboxylase 1 (ACC1) in both the adipose tissue and liver, and modulate differentially lipogenic enzymes such as sterol regulatory element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor-γ (PPAR-γ) in a tissue-specific manner. In addition, Vitamin D-VDR modulates fatty acid composition in subcutaneous adipose tissue through Elovl3 [35]. Therefore, combined with the previous research reports and the results of this study, we speculated that changes of maternal vitamin D status caused by 25-OH-D_3 supplementation might regulate milk fatty acid composition through altering gene expressions related to fatty acid synthesis in mammary gland. However, the underlying mechanisms are not clear and further studies need to be conducted to better understand the function of maternal 25-OH-D_3 supplementation in regulation of milk fatty acid synthesis, and the interaction between 25-OH-D_3 and key genes of fatty acid synthesis in mammary gland.

Bone is a dynamic and living tissue undergoing the process of remodeling. Biomarkers of bone remodeling are classified into bone resorption and formation markers, which reflect osteoclastic activity and osteoblastic activity, respectively [36, 37]. Among enzyme activity markers,

Table 5 Effects of dietary vitamin D_3 source on fatty acid composition of tibial and femoral cortical bones in piglets (g/100 g total fatty acids)

Items	Tibia			Femur		
	ND	25-D	*P* value	ND	25-D	*P* value
C12:0	0.197 ± 0.011	0.166 ± 0.015	0.10	0.148 ± 0.012	0.131 ± 0.009	0.19
C14:0	1.78 ± 0.10	1.60 ± 0.11	0.28	1.52 ± 0.06	1.35 ± 0.06	0.15
C14:1	0.072 ± 0.002	0.051 ± 0.011	0.11	0.058 ± 0.002	0.050 ± 0.005	0.04
C15:0	0.179 ± 0.007	0.175 ± 0.005	0.50	0.106 ± 0.006	0.096 ± 0.003	0.18
C16:0	31.54 ± 0.37	31.94 ± 0.47	0.52	30.49 ± 0.34	30.04 ± 0.30	0.35
C16:1	5.58 ± 0.29	4.95 ± 0.33	0.18	4.81 ± 0.24	4.35 ± 0.24	0.21
C17:0	0.341 ± 0.010	0.360 ± 0.013	0.32	0.265 ± 0.005	0.281 ± 0.009	0.15
C18:0	14.64 ± 0.41	14.84 ± 0.51	0.76	15.35 ± 0.43	14.98 ± 0.36	0.53
C18:1n-9	29.82 ± 1.01	30.60 ± 0.92	0.58	27.84 ± 0.81	28.78 ± 0.67	0.39
C18:2n-6	9.41 ± 0.43	8.99 ± 0.63	0.59	11.92 ± 0.34	12.74 ± 0.49	0.21
C18:3n-3	0.294 ± 0.019	0.292 ± 0.025	0.92	0.418 ± 0.024	0.476 ± 0.015	0.07
C20:0	0.253 ± 0.011	0.263 ± 0.006	0.42	0.241 ± 0.011	0.233 ± 0.004	0.48
C20:1	0.608 ± 0.045	0.585 ± 0.047	0.73	0.573 ± 0.042	0.537 ± 0.046	0.60
C20:3n-6	0.240 ± 0.032	0.237 ± 0.029	0.94	0.292 ± 0.017	0.309 ± 0.036	0.68
C20:4n-6	2.00 ± 0.31	2.00 ± 0.33	0.10	2.84 ± 0.14	2.43 ± 0.12	0.04
C22:0	0.366 ± 0.032	0.361 ± 0.043	0.90	0.360 ± 0.036	0.378 ± 0.053	0.78
C22:1n-9	0.429 ± 0.056	0.383 ± 0.045	0.54	0.179 ± 0.016	0.152 ± 0.017	0.32
C22:6n-3	0.086 ± 0.015	0.103 ± 0.017	0.46	0.164 ± 0.022	0.184 ± 0.042	0.71
SFA	49.30 ± 0.46	49.71 ± 0.57	0.59	48.47 ± 0.66	47.50 ± 0.44	0.25
MUFA	36.51 ± 1.12	36.57 ± 1.10	0.97	33.46 ± 0.86	33.87 ± 0.76	0.73
PUFA	12.03 ± 0.79	11.62 ± 1.00	0.75	15.63 ± 0.44	15.48 ± 0.60	0.55
n-6 PUFA	11.65 ± 0.75	11.23 ± 0.96	0.73	15.05 ± 0.41	15.48 ± 0.56	0.55
n-3 PUFA	0.380 ± 0.032	0.395 ± 0.040	0.78	0.582 ± 0.033	0.660 ± 0.047	0.20
n-6:n-3	30.93 ± 0.75	28.62 ± 0.59	0.04	26.09 ± 0.87	23.71 ± 0.80	0.07

Values were given as mean ± SEM, and data were the means of six replicates of one piglet per litter ($n = 6$)

SAT = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0

MUFA = C14:1 + C16:1 + C18:1 + C20:1 + C22:1

PUFA = C18:2 + C18:3 + C20:3 + C20:4 + C22:6

n-6:n-3 = (C18:2 + C20:3 + C20:4)/(C18:3 + C22:6)

BALP is regarded as one of bone formation markers [36], while TRAP is regarded as one of bone resorption markers [37]. Our results showed that 25-D sows had higher serum BALP activity on day 21 of lactation. In this study, we also found that piglets suckling 25-D sows had higher serum BALP levels on day 21 of lactation compared with those suckling ND sows. Elevated serum BALP level is associated with calcification process and commensurate with active bone remodeling [36]. The previous studies demonstrated a positive relationship between serum 25-OH-D_3 and IGF-I concentration [38]. Furthermore, IGF-I plays an important role in regulation of bone mineral density and maintenance of bone mass [39]. Our results showed that maternal 25-OH-D_3 supplementation significantly increased serum IGF-I concentration in sows and piglets at weaning. In general, our results suggested that dietary 25-OH-D_3 supplementation in maternal diets improved maternal and neonatal bone formation and ossification.

The process of calcium absorption is mostly conducted in the small intestine, which occurs transcellularly in the duodenum and paracellularly across the length of the small intestine via active and passive pathways, respectively [40]. 1,25-$(OH)_2$-D_3 is formed from 25-OH-D_3 in kidney under the catalysis action of renal 1α-hydroxylase. As indicated by the real-time PCR results, the mRNA expression of renal CYP27B1 was significantly higher in 25-D sows than ND sows. In addition, the mRNA expressions of duodenal VDR, TRPV6, and CaBP-D9k were significantly higher in 25-D sows than ND sows, which further contributed to a higher Ca absorption rate. These findings could partially explain higher Ca content and improved biomechanical properties found in tibias and femurs of 25-D sows. Intriguingly, increased gene expression of renal CYP27B1 existed concomitantly with increased CYP24A1 expression in this study. As physiological regulation of 1,25-$(OH)_2$-D_3, higher expression level of renal CYP24A prevents the excess accumulation

Table 6 Effects of vitamin D_3 source in maternal diets on bone properties of piglets

Items	ND	25-D	*P* value
Body weight at slaughter, kg	6.45 ± 0.07	6.49 ± 0.05	0.71
Tibia			
Weight, g	24.27 ± 1.02	23.95 ± 0.89	0.82
Length, mm	66.00 ± 0.97	67.00 ± 1.13	0.51
Fat-free dry weight, g	8.38 ± 0.43	8.34 ± 0.32	0.93
Ash content, %	43.60 ± 1.06	44.79 ± 0.80	0.38
Ca content, %	15.13 ± 0.41	17.03 ± 0.19	< 0.01
P content, %	7.51 ± 0.56	7.74 ± 0.54	0.76
PGE_2, ng/mg protein	8.59 ± 0.68	7.95 ± 0.76	0.55
Bone density, g/cm^3	1.34 ± 0.05	1.36 ± 0.07	0.83
Breaking force, N	795 ± 15	830 ± 11	0.09
Failure deflection, mm	3.69 ± 0.19	3.97 ± 0.17	0.31
Stiffness, N/mm	242 ± 7	238 ± 9	0.76
Absorbed energy, J	1.73 ± 0.05	1.83 ± 0.09	0.34
Femur			
Weight, g	31.20 ± 1.31	30.78 ± 1.14	0.81
Length, mm	72.32 ± 1.36	74.00 ± 1.03	0.35
Fat-free dry weight, g	11.18 ± 0.59	10.97 ± 0.38	0.77
Ash content, %	44.42 ± 1.22	44.94 ± 0.72	0.72
Ca content, %	15.85 ± 0.21	16.49 ± 0.25	0.08
P content, %	7.55 ± 0.54	7.76 ± 0.25	0.74
PGE_2, ng/mg protein	8.80 ± 0.51	7.11 ± 0.50	0.04
Bone density, g/cm^3	1.33 ± 0.03	1.34 ± 0.02	0.52
Breaking force, N	873 ± 5	895 ± 9	0.06
Failure deflection, mm	4.11 ± 0.15	4.37 ± 0.17	0.30
Stiffness, N/mm	264 ± 12	239 ± 12	0.16
Absorbed energy, J	1.84 ± 0.14	2.12 ± 0.20	0.27

Values were given as mean ± SEM and data were the means of six replicates of one piglet per litter ($n = 6$)

Ash, Ca and P contents are calculated by dividing the ash, Ca and P weights by fat-free dry weight of tibias and femurs

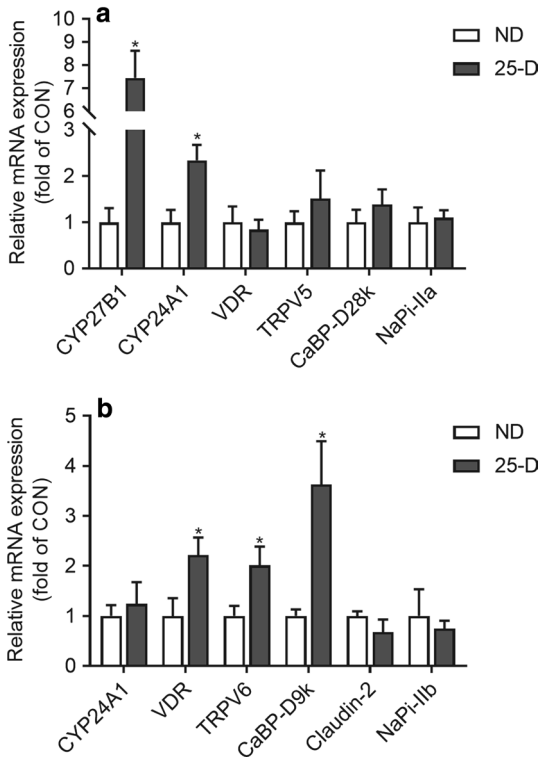

Fig. 3 Treatment effects on gene expression of sows. **a** mRNA expression of genes involved in Ca and P transport in the kidney. **b** mRNA expression of genes involved in Ca and P transport in the duodenum. Values were given as means ± SEM ($n = 4$). *$P < 0.05$ versus ND treatment

of toxic levels of the hormone, even when the expression of CYP27B1 is enhanced. Summarily, although urinary Ca loss cannot be ruled out, our study indicated that dietary 25-OH-D_3 supplementation significantly increased Ca absorption and reduced its fecal excretion in sows. In addition, piglets suckling 25-D sows had higher Ca content of tibias as well as an increased tendency toward femoral Ca content. These bone changes cannot be due to better growth, since the bone weights and lengths were not affected by the experimental diets. Therefore, combined with our previous results, the bone changes in piglets suckling 25-D sows must be caused by greater bone calcification. The duodenum has been the major focus of research due to its highly active transport system. However, it is the distal intestine where 70–80% of the ingested calcium is absorbed (mostly in the ileum) [41]. Probably, no more than 10% of total Ca absorption takes place in the large intestine, whether Ca intake is low or high [42]. Therefore, ileum and colon were selected as experimental objects to determine intestinal Ca transport of piglets during lactation. As indicated by the real-time PCR results, the mRNA expressions of VDR and claudin-2 were higher in the ileum of piglets suckling 25-D sows, whereas the mRNA expression levels of VDR and CaBP-D9k were increased in the colon of piglets suckling 25-D sows. The passive paracellular pathway dominates in the unweaned neonate and when calcium intakes are high, and the active transcellular pathway predominates in weaned and growing animals on a limited-calcium diet [40]. For newborn piglets, milk contains a relatively large and constant amount of Ca and P, and its composition seems to be independent of the content of these minerals in maternal diets [43]. High Ca intake leads to decrease of active transport, and passive absorption in jejunum and ileum is the primary process when Ca intake is adequate or high [42]. Ca absorption through the passive pathway is regulated by the tight junctions including claudin 2 and 12, which are specialized membrane domains mostly located in the apical region of intestinal epithelium [44]. Due to higher availability of Ca in milk [45], a little proportion of unabsorbed Ca in the small intestine could reach the large intestine. Therefore, it resulted in higher mRNA expression

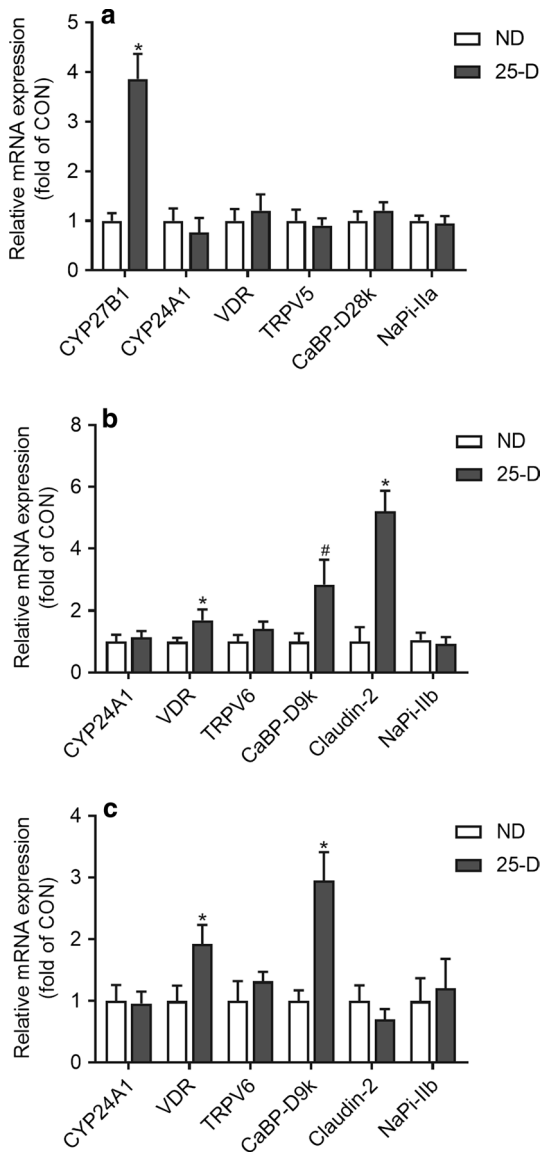

Fig. 4 Treatment effects on gene expression of piglets. **a** mRNA expression of genes involved in Ca and P transport in the kidney. **b** mRNA expression of genes involved in Ca and P transport in the ileum. **c** mRNA expression of genes involved in Ca and P transport in the colon. Values were given as means ± SEM ($n = 6$). *$P < 0.05$ versus ND treatment, #$0.5 \le P < 0.1$ versus ND treatment

of transcellular Ca transport-related genes in colon. Our study found that the expression of CYP27B1 and serum 1,25-$(OH)_2$-D_3 concentration were higher in piglets suckling 25-D sows at weaning. These changes caused elevated blood concentration of 1,25-$(OH)_2$-D_3 and further led to increase the expressions of key genes involved in transcellular and paracellular Ca transport.

In conclusion, our study suggested that maternal 25-OH-D_3 supplementation improved maternal and neonatal bone properties via modulating milk fatty acid composition and up-regulating mRNA expression levels of calcitropic genes. Application of maternal 25-OH-D_3 supplementation during lactation would be a fruitful area for improved bone health of mother–infant pairs.

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Author contributions The authors' contributions are as follows: XP and LZ designed research; LZ carried out the experiment design and wrote the draft of manuscript; LZ, JH, and ML were responsible for the data analysis; LZ, QS, and SL reviewed and revised the manuscript; XP had primary responsibility for final content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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