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Short Communication

Effect of 25(OH)D_3 Supplementation in Sows' Diets on Heart Development in Neonatal Piglets

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Authors' Contribution
QH, YL, HZ and ZT conceived and designed the experiments. JD, KH, YY and QL performed the experiments. MK, ZZ, NQ, YJ and SAF contributed reagents, materials, and analysis tools. HZ and QH wrote the manuscript.

Key words
25(OH)D_3, Ca^{2+}, IGF pathway, Myocardial cells, Piglets

ABSTRACT

The 25-hydroxyvitamin D [25(OH)D] level in serum is currently considered the best indicator of vitamin D supply to the body from cutaneous synthesis and nutritional intake. Few reports are available regarding the effects of vitamin D and its metabolites on heart development in swine. The effect of 25(OH)D_3 and Ca^{2+} supplementation in diets on heart development in neonatal piglets during pregnancy were examined in this study. Total 40 sows of 7 gestational age with good health and nutritional condition were divided into four groups (n=10): the control group, low calcium, 25(OH)D_3 group and low calcium with 25(OH)D_3 group. Each groups consist of 5 piglets, randomly selected and euthanized within 2 h after birth. After euthanasia, heart was collected for histopathological examination and IGF, IGF1, IGF2, IGFBP3, PCNA and ki67 expression were analyzed. The results showed that low calcium supplementation decreased the cardiac index and development of myocardial muscle fiber, while vitamins D_3 can improve the decrease of cardiac index and development of myocardial fiber due to low calcium supplementation. The expression profile of IGF1, IGF2, IGFBP3, PCNA and ki67 genes were down regulated in LCa group as compared to control group; while the vitamin D_3 supplementation significantly up regulated the above genes expression. In conclusion, our findings suggested that 25(OH)D_3 and Ca^{2+} supplementation in sows' diets has an important role in the development of heart and differentiation of myocardial fibers in neonatal piglets by changing the expression of the IGF pathway and myocardial cells proliferation.

Calcium is a major component of mineral in pigs, which makes up the bones and teeth and plays vital role in maintaining the normal functioning of nerves and muscle tissue (Gonzalo *et al*., 2018; Berchtold *et al*., 2000). The demand of calcium in pigs is necessary, and there is a harmonious relationship between calcium and phosphorus. Inadequate or inappropriate levels of calcium directly affect the normal growth, development and production of pigs (Gonzalo *et al*., 2018; Berchtold *et al*., 2000). Calcium deficiency in sows lead to birth paralysis, including prenatal and postnatal paralysis, characterized by muscle relaxation in the limbs, and low blood calcium during the prenatal and postnatal period (Tan *et al*., 2016). The low blood calcium in sows related to the various factors; a large amount of blood calcium enters colostrum before and after birth, resulting in a sharp decline in blood calcium; Stress of birth and decrease of intestinal absorption of calcium; lack of feed calcium phosphorus proportion, vitamin D deficiency, low magnesium diet may accelerate the occurrence of low blood calcium level (Braun, 1986; Tan *et al*., 2016; Yao *et al*., 2019).

Vitamin D_3 improve the absorption of calcium and phosphorus, saturate the plasma calcium and plasma phosphorus levels, promote growth and bone calcification (Yao *et al*., 2019; Braun, 1986). Vitamin D_3 is an essential nutrient for animals and has an important role in promoting the absorption of calcium and phosphorus from intestine (Braun, 1986; Yao *et al*., 2019). Moreover, 25(OH)D3 is more efficiently absorbed and has higher biological activity than vitamin D_3 (Amundson *et al*., 2017; Zhang *et al*., 2018, 2019). Previous study has shown that the circulating 25(OH)D_3 is the major indicator of vitamin D status in blood, and the concentration of 25(OH)D_3in cord blood is about 80% of that in maternal blood at birth (Zhang *et al*., 2019). However, only a small amount of 25(OH)D_3 from maternal blood could be supplied to piglets through breast

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milk. Supplementing 25-hydroxyvitamin D_3 can shorten the metabolic process of vitamin D_3 in the body and reduces the burden on the liver, but also avoids the impact on the absorption and utilization of vitamin D_3 due to intestinal injury, liver and kidney dysfunction (Zhang *et al*., 2019).

Ca^{2+} signaling is the basis for the growth and development of cells and organs (Gonzalo *et al*., 2018). As the earliest organ development, and functions in the embryonic development process, the morphological structure of the embryonic heart changed significantly, and the function of pumping blood increased continuously to adapt to the increasing physiological needs of the body (Zhang *et al*., 2019). From embryo to organ maturation, the function of cardiomyocytes changed dramtically, and the expression of calcium ion channels also changed significantly. Therefore, calcium plays an important role in the development and maturation of fetal porcine cardiomyocytes (Wullschleger *et al*., 2017).

Previous study has suggested that 25(OH)D_3 has a greater absorption efficiency as compared with regular vitamin D_3 (Zhang *et al*., 2019). However, there were few studies reported the relationship between maternal 25(OH)D_3 and Ca^{2+} supplementation during anaphase of embryonic development of maternal and neonatal. Meanwhile, potential regulatory effects of maternal 25(OH)D_3 and Ca^{2+} status during anaphase of embryonic development on heart development of neonatal piglets has not been evidently reported.

Materials and methods

Total 40 sows of 7 gestational age with good health and nutritional condition, similar genetic background and due date were selected, and randomly divided into 4 groups (n=10). The sows were kept and raised under the recommended temperature conditions. The feed was prepared as per recommendations of NRC while nutrient composition of the sow's diet is shown in Supplementary Table S1. The contents of calcium, phosphorus and 25(OH)D_3 in the diet were adjusted in accordance with the experimental design, and were divided into control group, low calcium group, 25(OH)D_3 group and low calcium with 25(OH)D_3 group each of 10 animals. The calcium, phosphorus and 25(OH)D_3 were supplied as shown in Supplementary Table S2.

These four groups, were raised from day 85 to day 110; after day 110, all the sows were fed in the delivery room with recommended temperature (20 °C). During the experimental period, the sows were feeding, drinking and immunization according to the husbandry procedures of the pig farm; meanwhile, the weight, feed intake and mortality in each group were recorded. A total of 5 piglets in each groups were randomly selected and euthanized within 2 h after birth. After euthanasia, the heart was collected and fixed in 4% paraformaldehyde; the body weight and the weight of the heart were measured for cardiac index analysis, and then the heart samples were dissected, immediately frozen in liquid nitrogen for RT-qPCR analysis.

The heart samples were fixed into 4% neutral buffered paraformaldehyde at 4 °C, and then tissue was dehydrated in graded (70%, 80%, 90%, 95%, and 100%) ethanol solutions, removed in xylene and embedded in paraffin. Sections were cut with 4-5μm thickness and placed on polylysine-coated slides for hematoxylin and eosin staining.

For *qRT-PCR* all the heart samples from each group were frozen in liquid nitrogen and homogenized in TRIzol reagent (TaKaRa) to extract total RNA, and reverse transcribed to cDNA by using a commercial kit (TransScript First-Strand cDNA Synthesis) with the recommended reaction temperatures. The qRT-PCR was performed five times by using the Step One-Plus™ Real-Time PCR System for IGF, IGF1R, IGF2, IGFBP3, PCNA, ki67 and GAPDH genes. The primers were synthesized by Shenggong (Shanghai) and are shown in Table I. The PCR reaction were performed with following thermal cycling parameters; denaturing at 98 °C for 30s, 40 amplification cycles at 98 °C for 10s, 57 °C for 40s and 72 °C for 40s.

The relative quantification

The data were expressed as mean ± standard deviation (SD) by using SPSS 19.0 software. $P<0.05$, statistically significant.

Table I. Primers used for the qRT-PCR.

Primers	Primer sequence (5' to 3')	Product size (bp)
IGF1	F: TTCTACTTGGCCCTGTGCTT R: CTCCAGCCTCCTCAGATCAC	222
IGF2	F: ACACCCTCCAGTTTGTCTGC R: GGGGTATCTGGGGAAGTTGT	212
IGF1R	F: ACTGTATGGTGGCCGAAGAC R: TCAGAGTGCGTGGTGAAGAC	163
IGFBP3	F: TCTGTCCACACCAAGATGGA R: GGAACTTGAGGTGGTTCAGC	181
PCNA	F: TGTGCTGGCAATGAAGACAT R: TCTCGGCATATACGTGCAAA	209
Ki67	F: CACCAGGCTTTACGGAAGAA R: AGATACGGGCTGCTTGAAAA	189
GAPDH	F: GTCGGTTGTGGATCTGACCT R: AGCTTGACGAAGTGGTCGTT	210

Results and discussion

Ca^{2+} is essential for a variety of vital physiological processes such as cell proliferation, differentiation, motility, secretion, excitation, and apoptosis. Adding suitable calcium and vitamin D_3 in the diet can improve the growth performance of animals significantly, such as reduced feed-meat ratio, increased daily weight gain and daily feed intake (Braun, 1986; Yao *et al*., 2019).

However, there were limited studies about the relationship between maternal 25(OH)D_3 and Ca^{2+} supplementation on heart development of neonatal piglets during anaphase of embryonic development of maternal and neonatal has been reported. In our study, the results indicated that the daily weight gain was increased in vitamin D_3 and LCa+VD_3 groups as compared with control and LCa groups from day 0-7d and 15-21d. Whereas, the daily weight gain was decreased in VD_3 and LCa+VD_3 groups (Fig. 1A). However, the average daily weight gain was no significantly changed among those four different treatment groups during the experimental period (Fig. 1B), which indicated no correlation to the average daily weight gain between 25(OH)D_3 and Ca^{2+} supplementation.

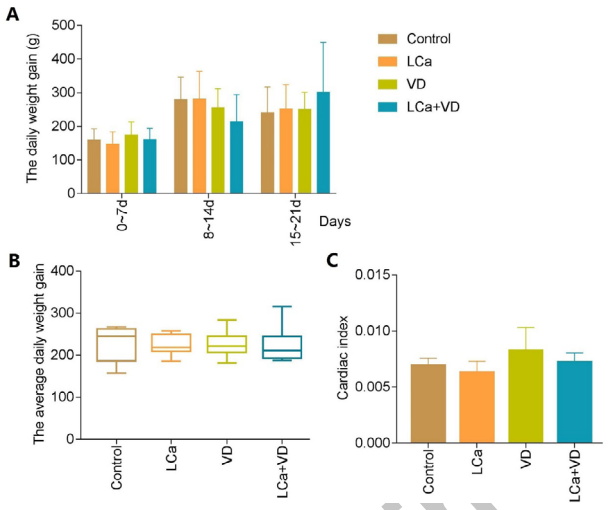

Fig. 1. Performance and cardiac index analysis of different treatment groups. Daily weight gains of different groups piglets in day 7, 14, 21 (A); Average daily weight gain (B); Effects of 25(OH)D_3 and Ca^{2+} supplementation in sows' diets on cardiac index (C).

The main hormones that regulate calcium and calcium metabolism are active vitamin D, parathyroid hormone and calcitonin (Peacock, 2010). Vitamin D promotes calcium absorption and bone salt deposition in the small intestine. The main target organs are the small intestine and bone (Yao *et al*., 2019; Amundson *et al*., 2017). The principal action of vitamin D in maintenance of calcium homeostasis is increased intestinal calcium absorption and mineralization of bone (Zhang *et al*., 2019; Braun, 1986). In neonatal developing cardiac cells, the relative mature sarcoplasmic reticulum plays a role as the main source of Ca required for contraction compared to that in the fetus. Calcium homeostasis in cardiomyocytes shows central role in the development of the heart during its early development (Gao *et al*., 2017). In our study, the effects of 25(OH)D_3 and Ca^{2+} on cardiac index of piglets showed that low calcium supplementation decreased the index of cardiac compared to control group, while VD_3 can improve the decrease of cardiac index, caused by low calcium. H and E stained histopathology micrograph showed that low calcium supplementation decreased the development of myocardial fiber, and VD3 can promote the development of myocardial fibers (Fig. 2).

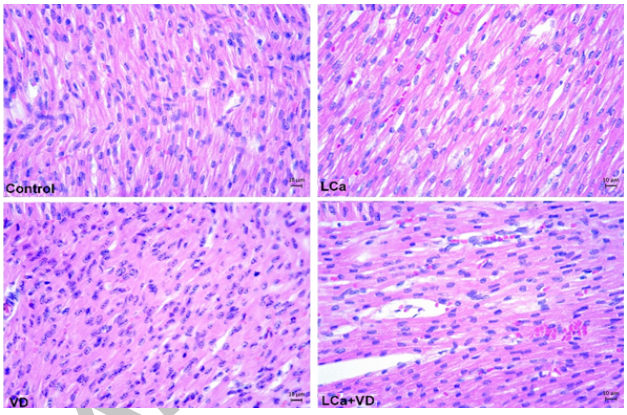

Fig. 2. Effect of Vit D_3 on histological structure of heart piglets. Note development of myocardial fiber A, control; B, low calcium treatment; C, Vit D_3 treatment; D, Vit D_3 + low calcium treatment. Stain: Hematoxilyn & Eosin.

As an important endocrine hormone, IGF has been confirmed to participate in variety of cell proliferation process, is mainly responsible for tissue growth and development, such as muscle, bone, kidney, skin, lung, and liver, etc. Meanwhile, as a strong mitogen, IGF can promote cell growth, proliferation, differentiation, and inhibits apoptosis (Du *et al*., 2019). The expression profile of IGF gene involved in the growth plates of piglets indicated that the mRNA expression of IGF was increased in LCa and LCa+VD_3 treatment groups as compared to control group and VD_3 group. However, the expression profile of IGF1, IGF2 and IGFBP3 genes were decreased in LCa group as compared with control group; while the VD_3supplementation can significantly up regulated the IGF1, IGF2 and IGFBP3 gene expression (Fig. 3).

Previous studies have shown that PCNA and ki67 are considered as the most widely used markers of cell proliferation (Jurikova *et al*., 2016). PCNA has significant roles in the metabolism of nucleic acid in DNA replication, DNA excision repair, cell cycle control, chromatin assembly, and RNA transcription; while ki67 is a nuclear antigen that exists in proliferating cells, connected with chromatin and related to cell mitosis (Jurikova *et al*., 2016). The mRNA expression of PCNA and ki67 genes were confirmed through qRT-PCR in control, LCa, VD and LCa+VD groups. The expression of PCNA and ki67 was decreased in LCa group compared to control and VD

groups. After the administration of VD, the expression of PCNA and ki67 was increased compared to LCa sows (Fig. 4).

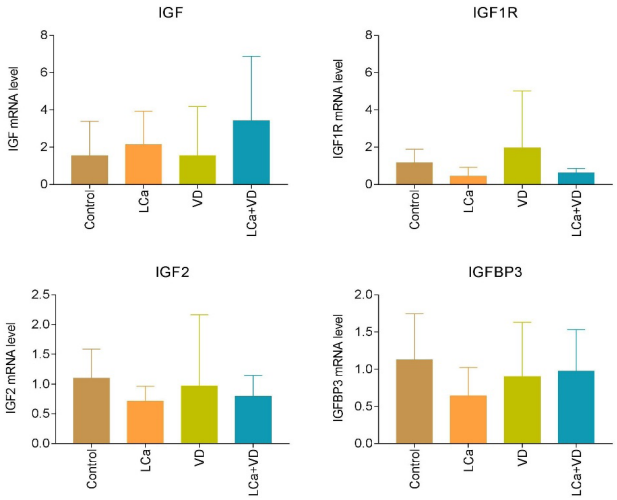

Fig. 3. Quantitative RT-PCR analysis of cardiac insulin-like growth factor (IGF). The mRNA expressions levels of IGF, IGF1R, IGF2 and IGFBP3 genes in the control, low calcium group (LCa), 25(OH)D_3 group (VD) and low calcium + 25(OH)D_3 group (LCa+VD) groups in the cardiac from piglets as determined by qRT-PCR. The data represent the mean ± SD.

In conclusion, our findings suggested that 25(OH)D_3 and Ca^{2+} supplementation in sows' diets shows key role in heart development in neonatal piglets, and 25(OH)D_3 treatment of gestational Ca^{2+} deficiency reactivates the IGF system and cell proliferation and promotes heart development and differentiation of myocardial fibers in the fetal pigs during late-gestation sows.

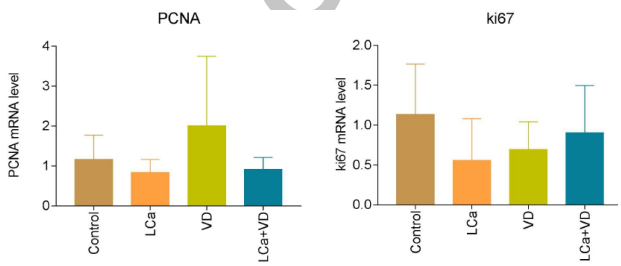

Fig. 4. Effect of 25(OH) D_3 and Ca^{2+} on PCNA and ki67 expression in neonatal piglets. *P< 0.05; expression levels were normalized to the levels of the geometric mean of GAPDH gene expression (mean ± SD).

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200528020558

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Effect of 25(OH)D_3 Supplementation in Sows' Diets on Heart Development in Neonatal Piglets

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Supplementary Table S1. Composition and nutrient levels of the basal diet.

Ingredients	Contents (%)	Nutritional level	Contents (%)
Corn (7.8%)	58.97	Dry material, %	87.33
Soybean meal (48%)	20.1	Sodium (Na), %	0.21
Wheat bran (16.5%)	10	Chlorine (Cl), %	0.301
Fish powder (67%)	3	Crude protein, %	18.527
Soybean	2		
Soybean oil	1.5		
Palm oil	1.5		
Premix *	1		
NaCl	0.4		
Total	100		

* The premix provided the following per kg of diets: V_A 10500 IU, V_E 70 IU, V_{K3} 3 mg, V_{B1} 3 mg, V_{B2} 7.5 mg, V_{B6} 4.5 mg, V_{B12} 0.03 mg, V_{B3} 30 mg, V_{B5} 15 mg, V_{B9} 1.5 mg, V_H 0.12 mg, V_{D3} 800 IU, Cu 20 mg, Fe 100 mg, Zn 100 mg, Mn 20 mg, I 0.08 mg, Se 0.30 mg and Cr 0.20 mg.

Supplementary Table S2. Composition levels of Ca/P and 25(OH)D_3 in the basal diet.

Groups	Ca/P (%)	25(OH)D_3
Control group (CG)	0.75/0.592	0 μg
Low calcium group (LCa)	0.65/0.513	0 μg
25(OH)D_3 group (VD)	0.75/0.592	50 μg
Low calcium + 25(OH)D_3 group (LCa+VD)	0.65/0.513	50 μg

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