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Effect of vitamin D supplementation during pregnancy on the Th1/Th2 cell balance of rat offspring

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Vitamin D has important functions in the immune system, and it may suppress the proliferation of T helper (Th) cells and modulate their cytokine production. In this study, we aimed to investigate the effects of maternal supplementation with different doses of vitamin D on the allergy status of the offspring. We gave pregnant female rats a low dose (48000IU/kg, equal to 800IU/d in human) and a high dose (240000IU/kg,equal to 4000IU/d in human) of vitamin D3 intramuscular injection on gestation day (GD)17, and we used an enzyme-linked immunosorbent assay (ELISA) to determine the levels of immune responsive cytokines including IL-4, IgE, and interferon γ (IFN-γ) in the offspring. On postnatal day (PND) 21, plasma IL-4 levels were elevated by 10.43% ($p < 0.01$) in the offspring from the high dose vitamin D3 group compared with the control group. And offspring plasma IL-4 levels in the low dose group decreased by 7.27% ($p < 0.05$) compared with the control dose group. We found that the offspring of mothers given a low dose of vitamin D3 had a 6.17% ($p < 0.01$) decrease in their plasma IgE levels compared to control animals, but the high dose of vitamin D3 showed no effect. The serum 25(OH)D3 levels were negatively correlated with the IL-4 ($r = -0.561$, $p < 0.01$) and IgE ($r = -0.421$, $p < 0.05$) levels of the offspring from the low dose group. In the lung tissues of the offspring of the high dose group, we observed thickening of the alveolar septa and more inflammatory cells compared with the control group and low dose group. Thickened alveolar septa were also found in the lung tissues of the offspring from the control group. We conclude that high dose vitamin D3 maternal supplementation during pregnancy induced an imbalance of Th1 and Th2 cells in their offspring resulting allergic and inflammatory response.

1. Introduction

Vitamin D is an important nutritional factor. Most vitamin D is endogenously produced by the action of sunlight on 7-dehydrocholesterol in the skin (vitamin D3), but it can also be obtained from diet (vitamin D3 or vitamin D2) (Holick et al. 1980). *In vivo*, vitamin D3 is metabolized in the liver to form 25-hydroxyvitamin D3 (25[OH]D), the main circulating form of vitamin D. This form can be converted to 1,25-dihydroxyvitamin D (1,25[OH]2D), the most biologically active form of vitamin D (DeLuca 2004). 1,25(OH)2D exerts multiple biological effects by binding to the nuclear vitamin D receptors (VDRs) that have been found in many tissues, including bone, the parathyroid gland, intestines, kidneys and lymphocytes (Maiya et al. 2008; Mawer and Davies 2001). 1,25(OH)2D has been considered important for maintaining calcium homeostasis by increasing calcium absorption and reabsorption in the urinary tract and intestines. It also participates in the regulation of parathyroid hormone (PTH) that increases calcium metabolism in a negative feedback loop (Wagner et al. 2012). A growing body of evidence suggests that vitamin D status is important to health, and vitamin D inadequacy has been implicated in the etiology of many diseases, including type I diabetes, tuberculosis, cardiovascular disease and some forms of cancer (Bjelakovic et al. 2011; Holick 2004). During pregnancy, especially during the last trimester, approximately 25–30 g of calcium is transferred to the fetus, and maternal 1,25(OH)2D requirements can increase up to four- or five-fold to meet the extra calcium needed for fetal skeletal growth (Mulligan et al. 2009; Specker 2004; Perez-Lopez 2007). Maternal vitamin D deficiency is a significant public health issue, and vitamin D supplementation during pregnancy could reduce the incidence of rickets, wheezing and type I diabetes during childhood (Holick 2004; Lucas et al. 2008). However, there has been a paucity of studies evaluating the requirements and effects of vitamin D supplementation during pregnancy. In 2011, the Institute of Medicine (IOM) recommended 600 IU per day in pregnant and lactating women, but the US Endocrine Task Force on Vitamin D commented that 1,500–2,000 IU vitamin D per day may be necessary to correct vitamin D deficiency in pregnant and lactating women (Ross et al. 2011; Holick et al. 2011)

VDRs were reported to be present on peripheral blood monocytes and activated T cells, suggesting a relationship between vitamin D and the immune system (Bhalla et al. 1983; Provve-

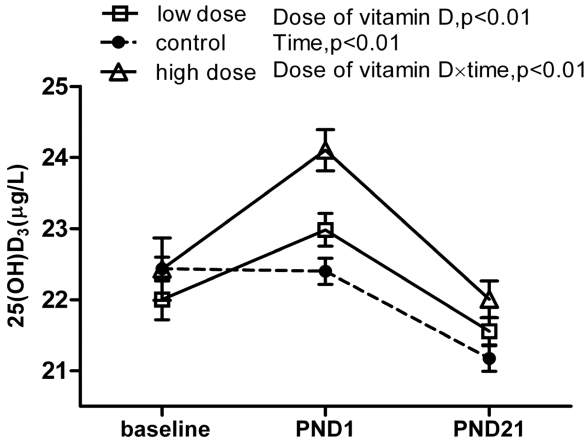

Fig. 1: Effect of vitamin D3 supplementation on the 25(OH)D3 levels of female rats Values are mean ± SEM (n = 6) A repeated measures ANOVA model was used to examine the effects of dose of vitamin D3 supplemetation, time and their interaction on serum 25(OH)D3 levels.PND: postnatal day.

dini et al. 1983). Recent experimental studies confirmed that T-cells are direct targets of 1,25(OH)2D3, and this interaction could suppress Th cell proliferation and modulate their cytokine production (Lemire et al. 1985). Therefore, many studies have investigated whether maternal vitamin D status influences the risk of their children having allergies or asthma, conditions that are characterized by the activation of Th2 cells. Nwaru et al. (2010) suggested that maternal vitamin D intake from food during pregnancy was negatively associated with the risk of their children developing food allergies by 5 years of age. However, Back et al. (2009) showed that children who were supplemented with vitamin D (> 13 μg/d) showed an increased risk of allergic asthma. Milner et al. (2004) also reported that early vitamin supplementation in children was associated with an increased risk of developing asthma and food allergies.

All of these data show that different doses of vitamin D during pregnancy can influence the allergy status of offspring, however, the foundational mechanism has not been adequately investigated. In the present study, we examined the levels of the immune-responsive cytokines, including IL-4, IgE, and IFN-γ, that reflect the Th1/Th2 cell balance to characterize the relationship between maternal vitamin D supplementation and the immune status of the offspring rats.

2. Investigations and results

2.1. 25(OH)D3 levels of female rats

The response of the serum levels of 25(OH)D3 of female rats to different doses of vitamin D3 administered during pregnancy is illustrated in Fig. 1. We found that the 25(OH)D3 levels of female rats were at the same baseline before mating, and the trends in the serum 25(OH)D3 were the same regardless of the dose of vitamin D3 used. During pregnancy, the 25(OH)D3 concentration first increased and then decreased in two vitamin D3 used groups. However, at PND1, the high dose of vitamin D3 increased the 25(OH)D3 serum levels of female rats by 7.6% ($p < 0.01$) and 4.5% ($p < 0.05$) compared with the control and low dose groups, respectively (Fig. 1). No significant effect of different doses of vitamin D3 was found on the serum 25(OH)D3 levels at PND21.

2.2. Biochemistry of offspring

The high dose vitamin D3 maternal supplementation had a significant effect on the bone density of the offspring compared to that of the control group (0.0248 ± 0.003 vs.0.045 ± 0.0145 g/cm^2, $p < 0.01$) and the low dose group (0.0247 ± 0.004vs.0.045 0.0145 g/cm^2, $p < 0.01$) (Fig. 2A). As expected, both the low and high doses of vitamin D3 given to the pregnant rats increased the serum 25(OH)D3 levels of the offspring by 9.1% ($p < 0.05$) and 17.83% ($p < 0.01$) (Fig. 2B), respectively, compared to the control group. There was no difference in the serum 25(OH)D3 levels of the offspring of rats given the low and high doses of vitamin D3 (Fig. 2B). The serum calcium levels of the offspring of the high dose group were elevated by 26.98% ($p < 0.01$) and 17.68% ($p < 0.01$) compared with those of the control and low dose groups, respectively (Fig. 2C), and the serum phosphorus levels in the offspring were decreased by 31.68% ($p < 0.01$) and 21.43% ($p < 0.05$), respectively (Fig. 2D). The low and high doses of vitamin D3 decreased the AKP levels in the offspring by 27.46% ($p < 0.01$) and 43.53% ($p < 0.01$), respectively (Fig. 2E), compared with the offspring of the control group. A one-way ANOVA analysis showed that the serum PTH levels of the offspring of the high dose group were decreased by 9.69% ($p < 0.05$) compared with the control group (Fig. 2F).

Table 1: Correlation between serum 25(OH)D_3 levels and inflammatory cytokine levels in the offspring of the low dose group

Parameters	Vitamin D	
	r	P
IFN-r	0.114	0.596
IL-4	−0.561	0.004**
IgE	−0.421	0.046*

r = correlation coefficient (n = 24) *$p < 0.05$ and **$p < 0.01$.

2.3. Relationship between 25(OH)D3 levels and inflammation

To investigate the effect of vitamin D3 maternal supplementation on immune dysregulation, we examined the expression of inflammatory factors in the offspring. The plasma IL-4 levels were elevated by 10.43% ($p < 0.01$) in the offspring of the high dose vitamin D3 group compared with the control group. However, the plasma IL-4 levels of the low dose group were decreased by 7.27% ($p < 0.05$) compared with the control dose group (Fig. 3A). We found that low dose vitamin D3 supplementation decreased the IgE levels of the offspring by 6.17% ($p < 0.01$), but high dose vitamin D3 supplementation had no significant effect on IgE levels compared with the control group (Fig. 3C). No significant difference in the offspring IFN-γ level was found among these three groups (Fig. 3B). We further analyzed the correlations between the serum 25(OH)D3 levels and these inflammatory factors in the offspring. The serum 25(OH)D3 levels were negatively correlated with the IL-4 (r = -0.561, $p < 0.01$) and IgE levels (r = -0.421, $p < 0.05$) of the offspring from the low dose group (Table 1). No significant relationship was found between 25(OH)D3 supplementation and IL-4 or IgE levels in the high dose group (Table 2).

2.4. Inflammatory response in the lungs

H&E staining of lung tissue was used to study the effect of vitamin D3 on the inflammatory response in the lungs. In the offspring of the high dose group, we can see alveolar septal thickening and more inflammatory cells compared with the

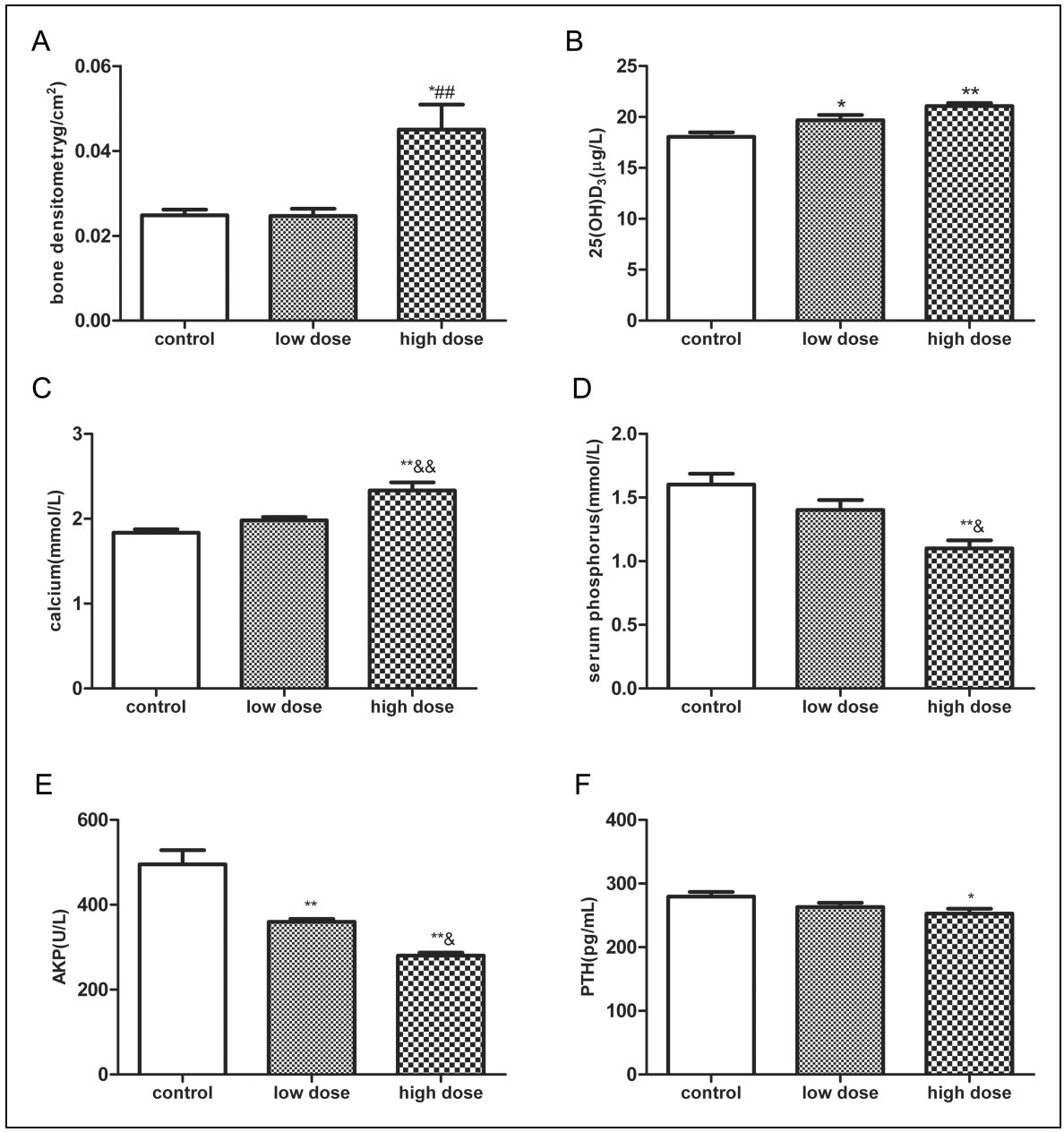

Fig. 2: Effect of vitamin D3 maternal supplementation on the biochemistry of the offspring A.bone densitometry; B. serum 25(OH)D3; C. serum calcium D. serum phosphorus; E. serum AKP;F. serum PTH. Values are mean ± SEM (n = 24) *$p < 0.05$ vs.control and**$p < 0.01$ vs. control; &$p < 0.05$ vs. low dose group and &&$p < 0.01$ vs. low dose group. Akp:alkaline phosphatase PTH:parathyroid hormone

control group and the low dose group. Septal thickening was also observed in the lung tissue of the control group offspring (Fig. 4).

3. Discussion

In this study, we found that vitamin D3 maternal supplementation could increase offspring serum 25(OH)D3 levels and

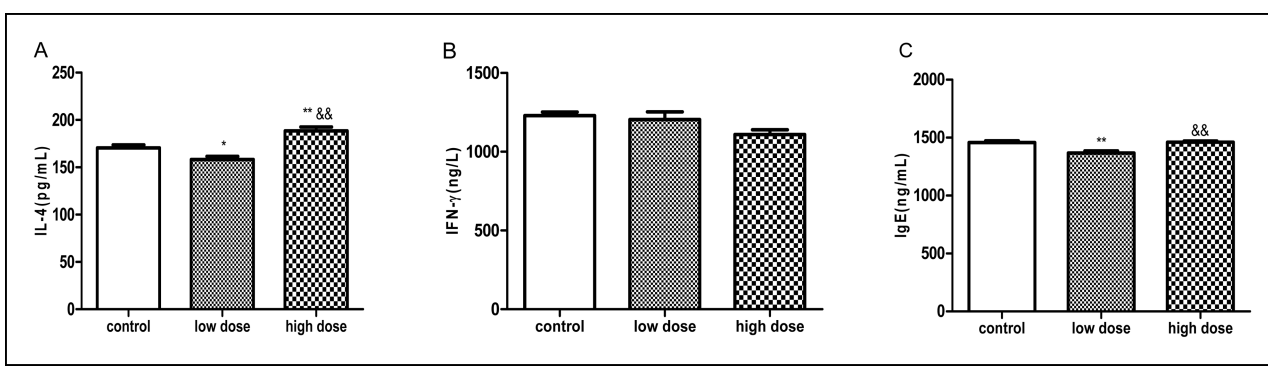

Fig. 3: Effect of vitamin D3 maternal supplementation on inflammatory cytokines in the offspring A.plasma IL-4; B. plasma IgE; C. plasma IFN-gamma Values are mean ± SEM (n = 24), *$p < 0.05$ vs. control, **$p < 0.01$ vs. control, and &&$p < 0.01$ vs. low dose group.

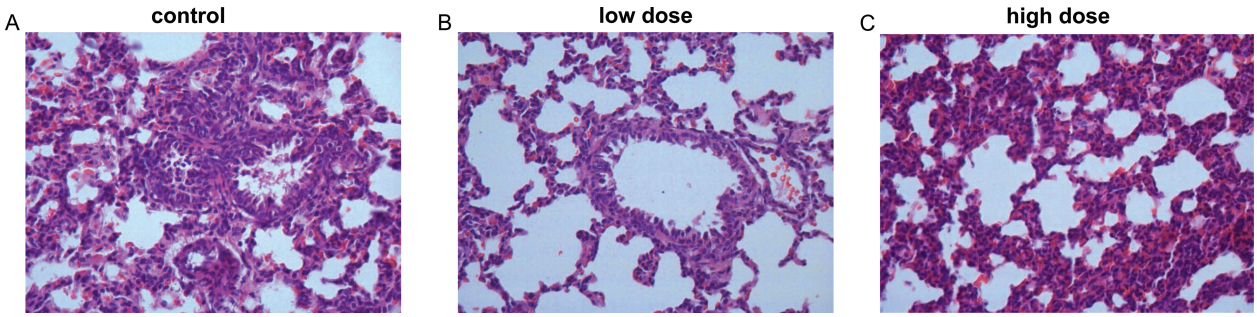

Fig. 4: Inflammatory cell infiltration in rat lungs Hematoxylin-eosin (H&E) staining of lung tissues. Magnification × 400. A.Offspring lung tissues from control group B.Offspring lung tissues from low dose group C.Offspring lung tissues from high dose group.

ameliorate calcium and phosphorus metabolism disorders. Our results also established that low dose vitamin D3 maternal supplementation could decrease offspring IL-4 and IgE level, and high dose vitamin D3 resulted in increased IL-4 in the offspring. Therefore, the data indicated that different doses of vitamin D3 supplementation during pregnancy have different effects on the immune state of offspring, and high dose vitamin D3 supplementation could induce a Th1/Th2 imbalance and stimulate the inflammatory response.

Recently, many clinical and experimental studies found that vitamin D could affect the manifestations of allergic diseases, however, there are no reports of the mechanisms by which vitamin D influences immunity. We gave pregnant female rats a low dose or a high dose of vitamin D3 via intramuscular injection. We found that supplementation with vitamin D3 could increase offspring serum 25(OH)D3 levels, and no significant difference in serum 25(OH)D3 levels was observed between the low and high dose offspring. Both low and high dose vitamin D3 supplementation increased the serum calcium levels of the offspring. Furthermore, the offspring serum phosphorus, AKP and PTH levels were lower in both of the vitamin D3 supplementation groups, and this result was in agreement with previous reports (Shah and Finberg 1994). The question then arose whether low and high doses of vitamin D3 maternal supplementation had different effects on the immune systems of the offspring. Early studies of vitamin D and the immune system suggested both T cells and B cells have VDR and that 1,25(OH)2D represses the proliferation of these cells (Provvedini et al. 1983; Lemire et al. 1984). Lemire et al. (1985) reported that 1,25(OH)2D can suppress Th cell proliferation and modulate the secretion of cytokines such as IFN-γ and IL-2 by Th1 cells and IL-4, -5 and -10 by Th2 cells. These cytokines play an important role in supporting cell-mediated and humoral immunity (Abbas et al. 1996).

In the current study, we tested the levels of IL-4 and IFN-γ, cytokines released by Th1 and Th2 cells, respectively, in the offspring of vitamin D-supplemented rats to examine the balance between Th1 and Th2 cells. The results showed that the low dose vitamin D3 maternal supplementation suppressed IL-4 secretion by Th2 cells in the offspring. The 25(OH)D3 levels were also negatively associated with the IL-4 levels in the offspring of the low dose group. The low dose vitamin D3 supplementation did not have any significant effects on IFN-γ production. We also found that the low dose of vitamin D3 ameliorated inflammatory cell infiltration into the offspring lung tissues. This observation is consistent with a report by Camargo et al. (2007) showing that high 25(OH)D3 levels during pregnancy decreased childhood wheezing by nearly 50% compared with low 25(OH)D3 levels. Erkkola et al. (2009) also showed that maternal vitamin D intake during pregnancy was negatively associated with the risk of asthma and allergic rhinitis in childhood. All of these studies indicated that low dose vitamin D3 supplementation during pregnancy could reduce the risk of Th2-mediated allergic airway disease and modulate the suppressive activity of local regulatory cells. However, much of the evidence was conflicting. Matheu et al. (2003) found that vitamin D treatment could augment allergen-induced T-cell proliferation, Th2 cytokine levels (IL-4 and IL-13) and IgE production in 5-week-old mice. Children whose mothers had a 25(OH)D3 concentration greater than 75 nmol/L during pregnancy had an increased risk of atopic eczema and asthma compared to children whose mother had a 25(OH)D3 concentration less than 30 nmol/L levels (Gale et al. 2008). Notably, we also found that high dose vitamin D3 maternal supplementation increased the offspring plasma IL-4 levels. Alveolar septal thickening and more inflammatory cell infiltration was found in lung tissues of the offspring of the control and high dose groups. In 2003, Matheu et al. reported the ability of vitamin D to alter the expression of Th1/Th2 cytokine and suggested that excess vitamin D could induce the development of a sustained Th2 response, leading to an increased risk of allergy, however, vitamin D might have beneficial effects against airway eosinophilia. Data of the present study indicate that high dose vitamin D3 maternal supplementation could improve offspring Th2 proliferation, stimulate IL-4 secretion and induce an imbalance in the Th1/Th2 ration, resulting inflammatory and allergic response. This different influence of IL-4 and IgE in the offspring may be decided by the dose and the time of vitamin D3 supplementation during pregnancy. Taken together, vitamin D3 maternal play an important role in inflammatory response and allergic disease of offspring, however, the mechanism by which vitamin D regulates immunity requires further investigation.

Interestingly, we found that serum IgE levels of the offspring of the control and high dose vitamin D3 maternal supplementation groups were higher than that of the low dose vitamin D group. IgE is a key player in the induction and maintenance of allergic inflammation and was considered be secreted by B lymphocytes in response to vitamin D (Hartmann et al. 2010). To illustrate the effect of different doses of vitamin D3 on the allergy status of offspring, we studied the lung tissues of the offspring and found that the high dose group had increased alveolar septal thickening and more inflammatory cells compared to the control and low dose groups. These data revealed that IgE and vitamin

Table 2: Correlation between serum 25(OH)D_3 levels and inflammatory cytokine levels in the offspring of the high dose group

Parameters	Vitamin D	
	r	P
IFN-r	−0.201	0.346
IL-4	−0.053	0.802
IgE	−0.208	0.340

r = correlation coefficient (n = 24) $*p < 0.05$ and $**p < 0.01$.

D have a complex dose-effect relationship as both no vitamin D and high vitamin D supplementation induced inflammatory cell infiltration, but low dose vitamin D inhibited the production of IgE. Hyponen et al reported that IgE concentrations were higher in study subjects with low 25(OH)D3 levels (<25 nmol/L) and with very high 25(OH)D3 serum levels (> 135 nmol/L), which suggested that vitamin D had a nonlinear relationship with IgE (Hypponen et al. 2009). Together, these results suggested that offspring IgE levels may have a U-curve relationship with the maternal vitamin D3 supplementation.

In summary, we showed that high dose vitamin D maternal supplementation during pregnancy induced an imbalance in the Th1 and Th2 cell ratio in offspring. It is necessary to supply vitamin D during pregnancy, but supplementation doses in excess of 240000IU/kg may increase the risk of allergy and asthma in offspring.

4. Experimental

4.1. Animals and reagents

Female Sprague–Dawley (SD) rats (n = 18) were provided by the Animal Department of Harbin Medical University Health Science Center. The animals were housed in pairs on a 12:12-h light-dark cycle in a temperature-controlled room at 22±2 °C. All animal care and experimental protocols were in compliance with the Animal Management Rules of the People's Republic of China (Ministry of Health, P.R. China, document no. 55, 2001) and the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the Animal Care Committee of Health Science Center, Harbin Medical University. Vitamin D3 was obtained from Sigma (St. Louis, Mo, USA). The alkaline phosphatase (AKP) assay kit was purchased from Nanjing Jiancheng Bioengineering Company (Nanjing, Jiangsu, China), and the calcium assay kit was from Biosino Bio-Technology and Science (Beijing). The PTH assay kit and enzyme-linked immunosorbent assay (ELISA) kit for rat 25(OH)D3,IL-4,IFN-γand IgE were obtained from Wuhan BOSTER Bioengineering (Wuhan, Hubei, China). Other chemicals and reagents were of analytical grade.

4.2. Mating, parturition, and offspring

The female rats were allowed to mate with males at a 2:1 ratio, and mating was confirmed by observing the vaginal smear. Female rats showing sperm in the smear were separated on the day of detection, and this was considered as gestation day (GD) 0. The day on which pups were born before 16:00 h was designated as postnatal day (PND) 0. Pups remained with their mothers until weaned at PND 21.

4.3. Preparation of animal model

Pregnant female SD rats were randomly divided into 3 groups (n = 6). The control group (control) received intramuscular injections of 0.5 ml/kg of 0.9% saline, the low dose vitamin D3 group (low dose) were given 48000IU/kg (equal to 800IU/d in human) vitamin D3 intramuscularly and the high dose vitamin D3 group (high dose) were given 240000IU/kg (equal to 4000IU/d in human) vitamin D3 intramuscularly. The vitamin D3 was injected at 9 a.m on GD17. Blood was obtained from the vena orbitalis posterior of female rats before mating to test the level of 25(OH)D3. At the completion of the study (at PND 21), two male and two female offspring from each litter were subjected to blood collection by cardiac puncture under light ether anesthesia and were then autopsied following excessive ether inhalation. The right femoral bones and lung tissues were harvested.

4.4. Blood chemistry

Blood was collected from each rat as described above, and the sera were obtained by centrifugation at 3000 rpm for 15 min at 4 °C for evaluation of AKP, calcium and phosphorus levels using an automatic clinical chemistry analyzer. The levels of IL-4, INF-r, IgE, 25(OH)D3 and PTH in the serum were assessed by ELISA kits purchased from Wuhan BOSTER Bioengineering (Wuhan, Hubei, China).

4.5. Bone densitometry

The bone mineral content (BMC; g) area (cm^2) of the right femur of rat offspring were measured by dual X-ray absorptiometry (DXA). A Hologic Discovery A densitometer (Hologic, Inc., Bedford, MA, USA) and small animal hi-res software version 12.3 were used to perform all measurements and analyses.

4.6. Histopathology

Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The embedded lung were cut into 5 mm sections, stained with hematoxylin–eosin (H&E) and examined by light microscopy.

4.7. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance of the pregnancy/parturition days and level of vitamin D3 administration was calculated using two-way ANOVA with Bonferroni post hoc tests. One-way ANOVA was used to compare more two groups followed by a Newman-Keuls multiple comparison test. The significance of the bone densitometry data were determined by a Kruskal-Wallis H test. A $p < 0.05$ was considered statistically significant.

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