Correlation of 25-Hydroxyvitamin D Levels in Serum vs. Breastmilk in Vitamin D-Supplementation Breastfeeding Women during Lactation: Randomized Double Blinded Control Trial

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Background: *Vitamin D deficiency in pregnancy and lactation increases the risk of adverse perinatal outcomes; however, although Vitamin D supplementation during pregnancy and lactation is recommended, suggested dose ranges vary.*
Objective: *To determine whether vitamin D3 1,800 IU/d supplementation in lactating mothers improves their vitamin D status and breast-feeding milk.*
Material and Method: *This was a randomized, placebo–controlled study of Thai pregnant women in their third trimester. A total of 76 Thai lactating mothers and their breast-fed infants were studied with maternal 25 Hydroxyvitamin D 25 (OH) D levels of 10-30 ng/ml determined using Liquid Chromatography Mass Spectrometry Tandem (LC-MS/MS). One group received vitamin D3 1,800 IU/d supplementation for 6 weeks, and members of the other group were given a placebo. 25 (OH) D level of colostum and 6-week serum from breast-fed milk were measured by High Performance Liquid Chromatography (HPLC). The data from the two groups were analyzed and compared.*
Results: *The mean ($\pm$SD) maternal age was 27.16$\pm$5.13 years, and mean body mass index (BMI) was 22.29$\pm$5.08 kg/m^2. At 6 weeks, maternal 25 (OH) D levels had increased significantly in the vitamin D group (VD) 68.30$\pm$15.40 nmol/L compared to 55.15$\pm$13.57 nmol/L in the placebo group ($p<0.001$) measured using the Liquid Chromatography-Mass Spectrometry Tandem (LC-MS/MS) method. Breast-fed milk did not show any significant incremental change in 25 (OH) D levels measured by High Performance Liquid Chromatography (HPLC); however, the change in 25 (OH) D levels in breast milk in the VD group was significantly different from that of the placebo group ($p = 0.005$).*
Conclusion: *Vitamin D3 supplementation during lactation can increase 25 (OH) D levels in Thai breast-fed mothers. Further work is needed to determine the duration of vitamin D supplementation to normalize breast milk and breast-fed infants' 25 (OH) D level at over 75 nmol/L.*

Keywords: *Breast-fed milk, Vitamin D supplementation, Lactation*

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In children, long-term overt vitamin D deficiency leads to rickets with significant skeletal deformities and poor growth. Additionally, low vitamin D status at birth and in infancy has recently been linked to an increased risk of acute lower respiratory tract infections including respiratory syncytial virus infections[1-3]. The natural sources of vitamin D in breastfeeding infants are previous placental transfer, human milk, and sunlight exposure. The vitamin D stores in the infant at birth depend on maternal vitamin D status during pregnancy and start physiologically with transplacental transfer of vitamin D as 25 (OH)

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D[4]. It is well-known that there is a positive correlation between maternal and cord blood 25 (OH) D concentrations, and generally, the cord blood 25 (OH) D level is between 50% and 80% of the maternal value[4]. Despite an abundance of sunshine, studies from the Middle East have shown a high prevalence of vitamin D deficiency in women of childbearing age, mainly attributed to cultural avoidance of sunlight, traditional dress styles covering most of the body, and inadequate vitamin D intake[5,6]. Because the vitamin D status of the mother during pregnancy is important in determining that of the infant at birth, the strong relationship between maternal and cord blood vitamin D status[4] and vitamin D deficiency during pregnancy should borne in mind, especially in breastfeeding mother.

In 2003, the American Academy of Pediatrics (AAP) published guidelines recommending that all children older than two months receive 200 IU of supplemental vitamin D daily[7]. Subsequently, in 2008, the AAP issued an updated recommendation that all infants, children and adolescents receive a minimum of 400 IU of vitamin D daily through diet or supplements[8]. Infants who are breastfed should receive 400 IU of supplemental vitamin D daily[9-13]. Risk factors for vitamin D deficiency in infants being exclusively breastfed without vitamin D supplementation are insufficient sunlight exposure and low maternal vitamin D during pregnancy.

Vitamin D drops, which are preferable for infants, are not available in Thailand; therefore, vitamin D deficiency or insufficiency may occur during pregnancy and may increase the risk of abnormal fetal growth and bone development. It is still not clear whether supplemental vitamin D during the lactation phase of exclusive breastfeeding can improve the vitamin D status. The aim of this study was to investigate whether vitamin D3 supplementation (1,800 IU/day) can improve breastfed serum of infants and breast milk 25 (OH) D level.

Material and Method

Two hundred pregnancies at the third trimester were enrolled in this study at Rajavithi Hospital, Bangkok. The protocol of this research was reviewed and approved by the Ethics Committee of Rajavithi Hospital (No. 97/2013) and the Ethics Committee of Queen Sirikit National Institute of Child Health, Bangkok, Thailand. All of the mothers delivered singleton infants at term (>37 weeks) and planned to breastfeed their infants for over 2 months postpartum. Mothers who had 25 (OH) D level <25 nmol/L and >75 nmol/L were excluded from this study. Healthy mothers were recruited from those who delivered at Rajavithi Hospital in Bangkok, Thailand between October 2013 and September 2014.

The primary objective of this study was to compare 25 (OH) D levels of breastfed infants' assigned vitamin D (VD) at 6 weeks postpartum with those given a placebo. The secondary aim was to compare levels of 25 (OH) D of maternal serum and breast milk of infants in the vitamin D and placebo groups at 6 weeks postpartum.

The vitamin D3 supplement pills used in this study were designed by the Vitallife Company in Bangkok and made from dried product of vitamin D3 of approximately 1,800 IU/capsule.

A nurse at the antepartum unit was assigned to register more than three hundred mothers into this study. After consent was obtained, mothers' serum levels of 25 (OH) D at the third trimester were checked and those who had 25 (OH) D levels between 25 and 75 nmol/L were included in the study. Seventy-two term infants of mothers with insufficiency vitamin D status were enrolled into this study after delivery at Rajavithi Hospital if breastfeeding was planned for more than 2 months. Registered officers randomly assigned vitamin D3 and placebo capsules to breastfed mothers randomized by a computer programme, and the obstetric physician was blinded to the participants' study arms. Cord blood, serum, urine and breast milk after delivery were collected from one hundred and two mothers. At the sixth week postpartum, 68 mother-infant pairs visited the postpartum clinic to complete demographic data and follow-up questionnaires, and mothers' blood, infants' blood (5 ml), breast milk and mothers' urine were collected. Eight mothers were excluded from the study because of inadequate breast milk volume, and twenty-six mother-infant pairs were lost to follow-up at the 6-week appointment. Fig. 1 depicts the study protocol.

Follow-up questionnaires were collected prospectively for the purpose of this study during the interview. These included socio-demographics, maternal skin color, maternal vitamin D supplementation and sunlight exposure behavior in mothers. During the study, mothers were contacted weekly by telephone by nurses who were experts in breastfeeding to ensure that infants were being breastfed and to resolve any breastfeeding problems until the 6-week postpartum visit. Sun exposure behavior was assessed by duration of sun exposure (hours/day) in the week prior to the interview. Both physicians and enrolled officers were

blinded to the study arms of all participants.

Blood samples were drawn by venipuncture from mothers and infants, and the mothers' urine and breast milk were collected and placed in the same container labeled with the date and the participants' names. The samples were frozen at -80°C and send to the Department of Medical Technology, Faculty of Allied Health Science, Thammasat University. Serum concentration of 25 (OH) D of both mothers and infants were measured by the LC-MS/MS technique. Breast milk 25 (OH) D levels were measured by the HPLC method, and vitamin D binding protein (VDBP) levels were measured by the ELISA method. Blood samples were available to measure serum intact parathyroid hormone (iPTH) using the chemi-luminescence assay method in Rajavithi Hospital for the same sample of mothers and infants.

Statistical analysis

The variables of the study were maternal 25 (OH) D breast milk levels, maternal 25 (OH) D levels, and infants' 25 (OH) D levels at delivery and 6 weeks postpartum. ANOVA was use to compare mothers and infants by group at the baseline and 6 weeks postpartum stages. We also compared selected maternal socio-demographic factors, dose of other source of vitamin D supplementation, sun exposure behavior, and mothers' skin color of the two groups using nonparametric statistical tests. Correlation was performed to assess the relationships among the changes in maternal vitamin D status, in breast milk vitamin D, and in breastfed infant vitamin D status.

Results

The two study groups were similar (Table 1). Gestational ages ranged from 37-41 weeks atenrolment. Mean (±SD) baseline serum 25(OH)D values were 56.1±13.9 nmol/L in mothers (range 25.4-87 nmol/L) and 24.8±12.1 nmol/L in infants (range 6.8-64.5 nmol/L). We found only Vitamin D binding protein at baseline that tested by HPLC method had different in both groups by $p<0.001$(Table 1). Postpartum maternal 25 (OH) D concentrations in the mothers and their infants at 6 weeks postpartum were positively correlated (r = 0.44; $p = 0.001$)

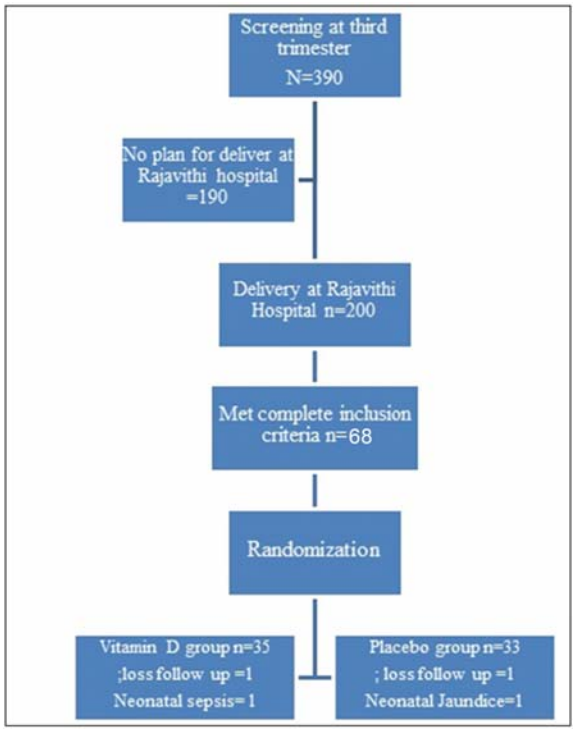

Fig. 1 Study protocol.

Table 1. General characteristics (n = 68)

Factors	vitamin D group(n=35)	placebo group(n=33)	*p*-value
Age (yrs)	26.14±5.04	28.24±5.08	0.092
Body weight (kg.)	57.03±11.27	52.64±12.74	0.136
Pre-gestational BMI (kg/m^2)	22.84±4.79	21.63±5.42	0.355
Pre-vitamin D status (nmol/L)	53.82±14.17	58.52±13.40	0.165
Breast milk vitamin D (nmol/L)	79.86±18.27	86.33±21.28	0.183
Vitamin D binding protein$^+$ (µg/ml)	172.08±6.44	179.55±7.26	<0.001*
Urine calcium (mEq/L)	6.19±6.82	6.09±8.54	0.990
Gestational age (weeks)	38.40±0.89	38.85±1.03	0.058
Male infants	18 (51)	17 (52)	0.593

Value are represented as Mean±SD and number (%)
$^+$ Test by ELISA method, * statistically significant at $p<0.05$

Table 2. Outcome after vitamin D supplementation during Lactation (6 weeks postpartum) (n = 68)

Factors	vitamin D group (n=35)	control group (n=33)	*p*-value
Maternal 25 (OH)D level at 6 weeks postpartum (nmol/L)	68.30±15.40	55.15±13.57	<0.001*
Change of maternal 25 (OH)D level (nmol/L)	14.21±15.88	-2.83±13.33	<0.001*
Breast milk 25(OH)D level at 6 weeks postpartum (nmol/L)$^{++}$	97.49±19.32	88.92±22.42	0.076
Change of 25(OH)D level in breast milk (nmol/L)	16.01±16.64	3.05±19.55	0.005*
Maternal urine calcium 6 weeks (mEq/L)	7.17±5.21	7.84±8.37	0.692
Maternal urine calcium/Urine creatinine 6 weeks	0.07±0.04	0.07±0.05	0.821

Values are represented as Mean±SD and number (%)
$^{++}$ Test by HPLC method, * statistically significant at $p<0.05$
Supplement data

Calcium level in mothers' serum and urinary calcium/creatinine ratio during study

	Placebo		1800 IU Vitamin D3/d	
	Visit 1	Visit 2	Visit 1	Visit 2
Serum calcium	9.23±0.34	8.86±0.89	9.22±0.34	8.65±0.68
Urinary Ca/Cr ratio	0.11±0.09	0.06±0.05	0.09±0.07	0.06±0.04

No statisticallt significant differences between groups at any visit. Hypercalcemia was defined as serum calcium over 10.5 mg/dL. Hypercalciuria was defined as calcium/creatnine ratio greater than 0.14.
Visit 1: postpartum Day 0, Visit 2: postpartum 6th week.

Maternal data and outcomes

Baseline maternal age, 25 (OH) D concentrations, vitamin D binding protein levels and gestational age were greater in the placebo group. Maternal serum 25 (OH) D values increased in the vitamin D groups from baseline to day 42 ($p<0.001$) (Table 2), and this incremental change in maternal serum 25 (OH) D was significantly greater in the vitamin D group than in the controls ($p<0.001$). Breast milk cholecalciferol concentrations mirrored serum concentrations, with increased values at 6 weeks postpartum in the vitamin D group ($p = 0.005$), (Table 2).

Correlation analysis (Fig. 2) showed additionally that change of breast milk 25 (OH) D levels was correlated to maternal 25 (OH) D change (r = 0.68, $p<0.001$).

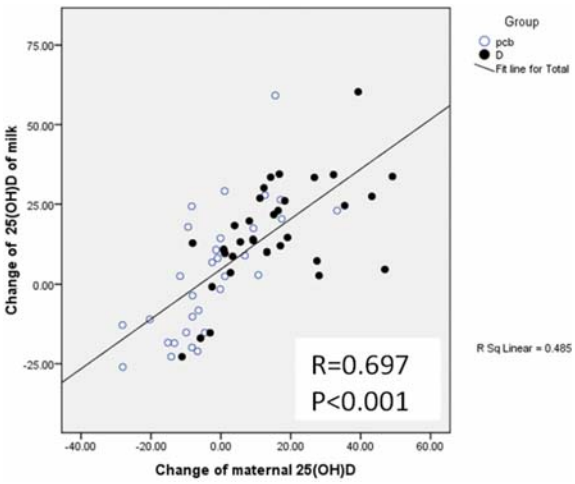

Fig. 2 Correlation of change of maternal 25 (OH) D, milk 25 (OH) D and infant 25 (OH) D (nmol/L) levels.

Discussion

The purpose of the present study was to evaluate the possibility of vitamin D supplementation of breastfed infants through their mothers. Prenatal and postnatal supplementation is advised for all infants in the Endocrine Society Clinical Practice Guidelines[14]. Previous studies have shown that high proportions of Malay and Thai pregnant woman are at risk of vitamin D deficiency[15,16] because, despite living in a country with plenty of sunshine, the majority of women in South East Asian countries had inadequate vitamin D levels

of 25 (OH) D (<75 nmol/L). A recent study[16] demonstrated that the prevalence of vitamin D inadequacy was 83.8%, 30.9% and 27.4% for first, second and third trimesters respectively. Vitamin D inadequacy is common in pregnant Thai women, and while vitamin D supplementation at 400 IU/day is likely to prevent vitamin D deficiency in mothers, it is unable to prevent vitamin D insufficiency status even at 800 IU/day. Our results showed maternal vitamin D insufficiency status in the placebo group was 56.1 nmol/L at delivery and 55.2 nmol/L at the 6-week postpartum period.

Our study suggests that vitamin D-supplementation, if large enough (1,800 IU/day), can improve 25 (OH) D in serum and breast milk in breastfed mothers. In a previous report, when mothers received 200 IU of vitamin D a day, the 25 (OH) D concentration of breastfed infants was almost equal to that of infants receiving 400 IU/day directly[17]. The maternal 25 (OH) D levels during the course of pregnancy showed a significant increase from the second to the third trimester[15,16].

As reported previously, 25 (OH) D levels are relatively unaffected by pregnancy despite the increase in calcitriol levels and the passage of 25 (OH) D across the placenta to the fetus[18].

Another study has suggested that human milk alone may provide sufficient dietary vitamin D for the needs of term infants under optimal social and environmental circumstances[19] . The dose of 900 IU of vitamin D daily[20] is not high enough to adequately boost breastfed infants' 25(OH)D levels, and even 1,000 IU/day is not sufficient[17]; on the other hand, a double-blind study in South Africa showed a clear improvement in infant 25(OH)D concentration with 500 IU of daily vitamin D supplementation through the mother[21]. Our results suggest that 1,800 IU of daily vitamin D3 supplementation in the mother can prevent vitamin D deficiency status in breastfed infants.

During an eight-week lactation period in another study, maternal 1, 25 (OH) D decreased to normal adult concentrations and remained quite constant thereafter[22]. In our study, six-week lactation showed a significant increase in breast milk 25 (OH) D levels in the vitamin D-supplemented group, although this may have been due to the lower levels of colostrum 25 (OH) D in the vitamin D group compared to the placebo group.

To check for possible side effects of vitamin D3 supplementation during breastfeeding, we monitored serum and urine calcium/creatinine ratios to avoid hypercalcimia (>10.5 mg/dL) and hypercalciuria (calcium: creatinine ratio >0.14). As shown in the supplementary data, we did not find any significant difference between the two study groups, and all parameters remained within the normal reference range. To check for possible side effects of vitamin D3 supplementation during breastfeeding, we monitored serum and urine calcium/creatinine ratios to avoid hypercalcimia (>10.5 mg/dL) and hypercalciuria (calcium: creatinine ratio >0.14). As shown in the supplementary data, we did not find any significant difference between the two study groups, and all parameters remained within the normal reference range.

This study had some limitations. Firstly, the number of participants was quite small and there were several complicated stages in the collection and storage of specimens, so that we were unable to show any correlation between the change of breast milk 25 (OH) D levels and the change of infant 25 (OH) D levels. Secondly, the method used to detect 25 (OH) D level in breast milk was HPLC and vitamin D binding protein level was test bythe ELISA method technique which may interfere with proteins in breast milk and serum showed data (Table 1) that disturbed results not in the same direct as LC-MS/MS technique. We had recommend LC-MS/MS technique for biomarker testing in pregnancy during pregnancy and breastfeeding period. This study, was well designed to test the hypothesis and to study correlations among maternal breast milk and infant 25 (OH) D levels in the vitamin D-supplemented group and the placebo one.

Conclusion

Vitamin D in breast milk is not sufficient to represent a secure way of ensuring vitamin D sufficiency status in Thai infants. Supplementation of vitamin D3 to the breastfed infant is not adequate to achieve 25 (OH) D sufficiency status. Further, more detailed studies are needed, as this dose of vitamin D3 supplementation is quite high for lactating mothers, and it has not previously been recommended for Thai lactating mothers.

What is already known on this topic?

Vitamin D deficiency in pregnancy and lactation carries an increased risk of adverse perinatal outcomes. There is a high prevalence of vitamin D deficiency in Thai pregnant women despite the abundance of sunshine in the country. Vitamin D Supplementation of 400-2,000 IU/day is recommended in all pregnancies.

What this study adds?

Vitamin D insufficiency status has high prevalence rates in Thai pregnancies. Vitamin D3 supplementation of 1,800 IU/day can improve the vitamin D insufficiency status of Thai pregnant females. Vitamin D3 supplementation may improve vitamin D levels in breast milk for breast-fed infants.

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Potential conflicts of interest

None.

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การศึกษาแบบปิดสองชั้นแบบสุ่มของระดับวิตามินดี ในเลือดแม่และนมแม่จากการเสริมวิตามินดีในช่วงให้นมลูก

สถิตย์ นิรมิตรมหาปัญญา, สุรศักดิ์ เก้าเอี้ยน, วราภรณ์ แสงทวีสิน, อนุสรณ์ พัฒนประภาพันธุ์, นริสา บดีรัฐ, ชัยชาญ ดีโรจนวงศ์

ภูมิหลัง: *การขาดวิตามินดีในช่วงตั้งครรภ์และให้นมบุตรจะเพิ่มความเสี่ยงต่อสุขภาพเด็กในช่วงหลังคลอด จึงแนะนำให้มีการเสริมวิตามินดีในหญิงตั้งครรภ์และให้นมบุตรแต่ขนาดของวิตามินดีที่ให้มีความแตกต่างกันมาก*

วัตถุประสงค์: *เพื่อศึกษาการเสริมวิตามินดี 3 ที่ขนาด 1,800 ยูนิทต่อวันในหญิงที่ให้นมบุตรจะสามารถเพิ่มระดับวิตามินดีของเลือดและน้ำนมแม่*

วัสดุและวิธีการ: *การศึกษาทดลองแบบปิดฉลาก 2 ชั้นในหญิงไทยตั้งครรภ์จำนวน 76 คนที่มีระดับวิตามินดีอยู่ระหว่าง 10-30 นาโนกรัมต่อมิลลิลิตรในช่วงไตรมาสสุดท้ายของการตั้งครรภ์แล้วเสริมด้วยวิตามินดี 3 ที่ขนาด 1,800 ยูนิทต่อวัน ในช่วงให้นมบุตร โดยวัดระดับวิตามินดีของเลือดแม่ตรวจด้วยวิธี LC-MS/MS และระดับวิตามินดีของน้ำนมแม่ ที่หลังคลอดทันทีและที่ 6 สัปดาห์ด้วยวิธี HPLC โดยแบ่งกลุ่มเป็นแบบเสริมและไม่เสริมวิตามินดี ซึ่งนำข้อมูลมาวิเคราะห์เปรียบเทียบทั้ง 2 กลุ่ม*

ผลการศึกษา: *ค่าเฉลี่ยของอายุแม่ 27.16±5.13 ปี, ดัชนีมวลกายเฉลี่ย 22.29±5.08 กิโลกรัมต่อตารางเมตร พบว่าการเสริมวิตามินดี 3 เป็นเวลา 6 สัปดาห์ สามารถเพิ่มระดับวิตามินดีในเลือดแม่ได้อย่างมีนัยสำคัญทางสถิติ ($p<0.001$) แม่ที่ได้รับการเสริมวิตามินดีจะมีระดับ 25 (OH) D ที่ 68.30±15.40 นาโนโมลต่อลิตร ขณะที่แม่ที่ไม่ได้รับการเสริมวิตามินดีจะมีระดับ 25 (OH) D ที่ 55.15±13.57 นาโนโมลต่อลิตรในกลุ่มยาหลอก แต่ไม่พบความแตกต่างของระดับวิตามินดีในนมแม่จากการตรวจด้วย HPLC แต่การเปลี่ยนแปลงของระดับวิตามินดีในนมแม่เพิ่มขึ้นอย่างมีนัยสำคัญในแม่ที่ได้รับการเสริมวิตามินดี ($p = 0.005$)*

สรุป: *การเสริมวิตามินดีในช่วงให้นมบุตรสามารถเพิ่มระดับวิตามินดีในเลือดแม่ได้และอาจจะส่งผลถึงการเพิ่มระดับวิตามินดีในนมแม่ด้วย ควรมีการศึกษาเพิ่มเติมถึงช่วงเวลาที่ให้เสริมวิตามินดีจนทำให้ระดับวิตามินในนมแม่และเลือดเด็กมีค่าปกติได้ที่ 75 นาโนโมลต่อลิตร*