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ASSOCIATION OF DECREASED SERUM 25-HYDROXY VITAMIN D3 LEVEL WITH RECURRENT PREGNANCY LOSS: A PROSPECTIVE COHORT STUDY IN BANGLADESHI WOMEN

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ABSTRACT

Background: Recurrent pregnancy loss (RPL) is one of the frequently occurring infertility related problem world-wide. Low serum 25-hydroxyvitamin D3 (25(OH)D3) concentrations may be associated with pregnancy loss. To justify this proposition, we studied the serum 25(OH) vitamin D3 concentrations in pregnant women who have experienced two or more pregnancy loss and control subjects who had successful pregnancy history. **Objective**: In this study, we investigated whether serum 25(OH)D3 concentration was associated with recurrent pregnancy loss. **Design**: In this prospective cohort study, 45 pregnant women in their early pregnancy with previous history of two or more early spontaneous pregnancy loss and 45 women in their early live pregnancy with previous history of one or more successful pregnancy and no history of any pregnancy loss were recruited to investigate the association between maternal serum concentrations of serum 25(OH)D3 and the risk of experiencing pregnancy loss (n = 45). **Results**: Our study revealed that serum 25(OH)D3 concentration is significantly lower in RPL group than the healthy controls (P<0.01). The multivariable logistic analysis revealed that serum 25(OH)D3 concentration is negatively associated with RPL outcome (OR: 0.78 with 95% CI: 0.62, 0.99) with 22% lower odds for one extra level of serum 25(OH)D3 concentration. We also found that pregnant women who does not intake vitamin D3 containing food has 5.6 times higher odds of having RPL outcome compared to those who intakes vitamin D3 containing food (OR: 5.66; CI: 1.56, 20.52). **Conclusions**: We found an association between serum 25(OH)D3 concentration and RPL, which indicates that serum vitamin D3 acts as a modifiable risk factor for pregnancy loss.

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INTRODUCTION

Recurrent pregnancy loss (RPL) is defined as the loss of pregnancy for at least two times before the 20th week of gestation, which is one of the most frequently occurring human infertility-related disease affecting ~0.8% to 1.4% of women in the general population (1-3).

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Several factors are responsible for the occurrences of RPL, such as parental and embryonic chromosomal abnormalities, prothrombotic states, uterine structural diseases, endocrinological dysfunctions, infections and immunological factors (4). Despite the recent advances in the clinical and biochemical diagnosis of human infertility, the underlying causes of the 35% to 60% of RPL women remain unexplained, which indicates that genetic, epigenetic, nutritional and environmental factors may be responsible for the RPL phenotype (4-6). Vitamin D, a steroid prohormone, has a documented classical role in the regulation of bone health physiology and mineral homeostasis (7). Several recent lines of evidences showed that physiologically active form of vitamin D, 1,25 dihydroxyvitamin D3 (1,25(OH)2D3)

has strong immunomodulatory effects on various tissues (8-10), which has led to hypothesize that vitamin D might have prominent role in pregnancy (11). Subsequent findings of vitamin D receptor (VDR) and the enzymes involved in hydroxylation of vitamin D involved in localized vitamin D synthesis in human placenta and deciduas have further implicated the importance of this hormone in reproductive function and considered as a potent risk factor for recurrent pregnancy loss (12-15). This was further justified by the gene expression study which revealed that women with RPL had a significantly lower expression of VDR and CYP27B1 mRNA and protein in villous and deciduas tissues in compared with the normal pregnant women (16,17). Moreover, poor plasma vitamin D level during pregnancy has been reported to be associated with preeclampsia (18), gestational diabetes (19,20), bacterial vaginosis (21) and compromised intrauterine growth (22). In addition, decreased level of serum vitamin D was shown to be associated with recurrent pregnancy loss in several studies (13,14,23-25). However, two studies did not find any association between serum vitamin D status and recurrent pregnancy loss which has confounded the role of vitamin D3 in association with RPL and raised the urgency for further clarification (26,27). Vitamin D deficiency can be defined as serum levels of 25- hydroxyvitamin D < 20 ng/mL and insufficiency as levels between 21 and 29 ng/ mL according to the Endocrine Society of Clinical Practice guidelines (28). Vitamin D deficiency or insufficiency is a common problem in pregnant women world-wide (29-33). However, certain risk groups such as populations with darker skin, people who have minimal sun exposure or specific dietary habits, such as vegetarianism are more prone to have lower levels of serum vitamin D (32,34). To date, no study is available on the association of serum vitamin D3 concentration and RPL outcome in Bangladeshi women who has comparatively darker skin and reduced sunlight exposure due to traditional clothing which covers whole body. In order to further clarify the role of serum 25-hydroxy vitamin D3 in recurrent pregnancy loss, we recruited 45 women with 2 or more pregnancy loss and 45 women with healthy pregnancy in this cross sectional study and investigated the association between serum 25-hydroxy vitamin D3 and vitamin D containing food intake with risk of pregnancy loss.

METHODS AND MATERIALS

Study Subjects: The study was approved by the ethical review committee of Bangabandhu Sheikh Mujib Medical University. An informed written consent was taken from all the participants upon recruitment. This cross-sectional study included 45 pregnant women in their early pregnancy (documented by ultrasonography) with previous history of two or more early spontaneous pregnancy loss and 45 women in their early live pregnancy (documented by ultrasonography) with previous history of one or more successful pregnancy and no history of any pregnancy loss. Both subjects were recruited from the outpatient Department of Obstetrics & Gynecology, BSMMU. Subjects with known cardiac disease, renal dysfunction, kidney stones, rheumatoid arthritis, arteriosclerosis, thyroid, parathyroid, or adrenal disorders, type I diabetes, infertility, known malignancy, malabsorption syndrome, genetic abnormalities and Cushing syndrome were excluded from the study. All the subjects who had regular menstrual cycles and had not taken hormones or vitamin D for at least 3 months were included in the study. Body mass index (BMI) was measured from their weight and height recorded by a nurse during the study. In addition, a general questionnaire including the participants' socio-demographic characteristics and information about prior abortion, smoking, alcohol consumption, sunscreen usage, diseases, medications, and intake of vitamin D containing of food was accomplished through face-to-face interview.

Estimation of serum 25-hydroxy vitamin D3: Approximately 5 ml whole blood was collected by venipuncture in a non-EDTA tube after taking informed written consent from respondents who fulfill the inclusion criteria. Serum was separated from the whole blood by centrifugation for 10 min at 3000 r.p.m. and serum 25-hydroxy vitamin D3 was measured on the same day to avoid inter-assay variation. Quantitation of serum 25(OH)D3 was performed by enzyme immunoassay using commercial EIA kits (Immunodiagnostics Systems, UK) according to manufacturer protocol. The absorbency of the samples was taken at 450 nm using a microplate reader (Micro Read 1000, ELISA plate analyzer, Global Diagnostics, Belgium).

Statistical analysis: All continuous variables were summarized in terms of means±SD and categorical variables in terms of percentage. Serum 25(OH)D3 levels were measured by CLIA three times and expressed as mean±SD in ng/mL. To examine the associations of potential risk factors (continuous variables) with disease outcome nonparametric test was performed that were not normally distributed and student's t-test was performed when normality assumption of the data was satisfied.

Chi-square test was employed to assess the association between qualitative variables and the study groups (disease outcome). Finally, the association of serum 25(OH)D3 with disease outcome was explored using box plot and student's t-test and multivariable logistic regression analysis was performed to examine the net effect of serum 25(OH)D3 on the disease outcome after adjusting for the other variables. For all tests, a probability (P) of <0.05 will be considered as significant. All statistical analysis was performed using the SPSS software version 20 (SPSS, Chicago, IL, USA) and Stata version 14.

RESULTS

Sociodemographic characteristics of the study subjects: A total of 90 women were included in this study with the age range of 20–33 years (Table 1).

Table 1. Comparison of the physiological characteristics of the study subjects

Variables	RPL	Control	*P* value
Age	26.83±4.32	26.06±3.54	0.418^{ns}
Weight (Kg)	56.80±9.42	56.2±7.27	0.797^{ns}
Height (cm)	152.91±4.49	153.43±5.24	0.657^{ns}
BMI	24.37±3.87	24.23±2.99	0.866^{ns}
Education			
Illiterate	11.4%	2.9%	0.333^{ns}
0-5years	14.3%	11.4%	
6-10years	37.1%	34.3%	
10-12 years	11.4%	28.6%	
Others	25.7%	22.9%	
Socio economic status			
Lower-middle (6827-26851)	71.4%	71.4%	
Upper- middle (26858-83018)	28.6%	25.7%	0.333^{ns}
High (>83018)	0.0%	2.9%	
Sunlight exposure			
<½ hour	62.9%	54.3%	
½ -1 hour	37.1%	40.0%	
1-2 hours	0.0	5.7%	
2-3 hours	0.0	0.0	
>3 hours	0.0	0.0	
Whole Body covered with cloths (more than 90%)			
	80.0%	88.6%	
	20.0%	11.4%	0.324^{ns}

BMI: Body mass index
Values are expressed as means±SD
ns=not significant
p value reached from Chi-square test

Among 90 women, 45 were for RPL group (26.83±4.32 years of age) and 45 were normal subjects with successful pregnancy (26.06±3.54 years of age). Table I shows the distribution of the study patients by age.

Table 2. Distribution of the study patients by vitamin D containing food (n=90)

Vitamin D containing food	Group I (n=45)		Group II (n=45)		P value
	n	%	n	%	
Yes	29	54.3	41	57.1	<0.001
No	16	45.7	4	42.9	

ns=not significant
p value reached from Chi-square test
Group I= Pregnant women in their early pregnancy.
Group II= Women in their early live pregnancy with previous history of one or more successful pregnancy and no history of any pregnancy loss.

The differences was statistically not significant (p>0.05) between two groups. Table 2 suggests that two groups had no significant differences with regard to distributions of general characteristics including age, education, employment status, body mass index and sun exposure. However, education level and monthly income had significant role in RPL outcome. Our study indicated that illiterate women are more prone to RPL than the educated women and high income women had reduced risk of developing RPL than low income women. Vitamin D containing food intake has shown to be another significant factor for the development of RPL outcome. Pregnant women who had more frequent intake of vitamin D containing food had reduced risk of manifesting RPL outcome than pregnant women with less frequent intake of vitamin D containing food (Table 3).

Table 3. Adjusted odds ratio from multivariable logistic regression

Group	Odd ratio	Standard error	z	P>[z]	95% confidence interval	
Serum 25(OH)D3	0.78315	0.09353	-2.05	0.041	0.6197	0.98970
D3 containing food	5.6599	3.7196	2.64	0.008	1.5610	20.5215
Constant	1.5529	2.2475	0.30	0.761	0.1910	26.4909

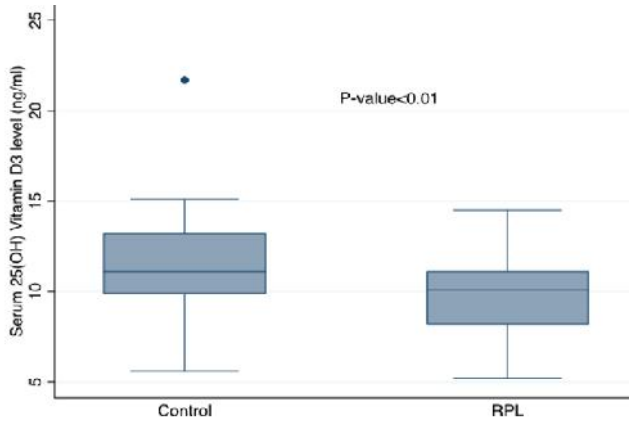

Figure 1. Serum 25(OH) vitamin D3 concentration in RPL and control subjects (n=90). Data are represented as mean±SD

The serum levels of 25(OH) D: The 25(OH)D3 levels were estimated in all women in the study. The mean 25(OH)D3 concentration was 9.90±2.28 ng/ml (range from 5.2 to 14.5 ng/ml) for women with RPL, in compare to control group with the median 25(OH)D3 level of 11.42±3.96 (range from 5.6 to 21.7 ng/ml). Figure 1 shows the mean distribution of the study patients by vitamin D level. It was observed that all (100.0%) patients had deficiency <20 ng/ml in group I and 44 in group II. The ANOVA test of 25(OH)D3 revealed that significant level of differences exist between the two groups (P=0.000). There was a statistically significant difference between the RPL group and the healthy pregnant group, (χ2 and P value of 13.416, 0.000 and 52.267, 0.000 respectively). There was a strong association between low vitamin D levels and RPL table 3 (odd ratio (OR): 0.7831;' 95% CI: 0.62, 0.99).

DISCUSSION

Even though the recent advancement of clinical and molecular diagnostic approaches, underlying causes of RPL remains unanswered for 50% of cases. Among the various etiological agents, vitamin D has now drawn great attention due to its role in autoimmunity and immuoregulation which is considered as an important risk factor for (23,34). Recent studies have shown that vitamin D promotes a more convenient environment for pregnancy by various mechanisms, such as differentiation of immune cells, cytokine production and enrichment of the Th2 cells mediated immunity (3,23,34,35). Vitamin D deficiency in pregnant women has demonstrated to be associated with increased risk of obstetrical problems, such as preeclampsia, bacterial vaginosis, preterm delivery and gestational diabetes mellitus (18-22). Therefore, it is assumed that vitamin D deficiency plays a major role recurrent pregnancy loss (35).–In this study, we analyzed the association between serum 25(OH)D3 concentrations and the risk of recurrent pregnancy loss. We demonstrate that women with recurrent pregnancy loss have lower serum concentrations of 25(OH)D3 in compared with the control subjects. We also found that frequent intake of vitamin D containing foods is negatively correlated with the recurrent miscarriages.

The mean serum concentration of 25(OH)D3 was 9.90±2.28 ng/ml in the women with two or more pregnancy losses, whereas women with successful pregnancy have 11.42±3.96 ng/mL. According to Endocrine Society of Clinical Practice guidelines, vitamin D deficiency is defined as serum concentration of 25- hydroxyvitamin D3 less than 20 ng/mL and insufficiency as levels between 21 and 29 ng/ mL (28). In our study, we found widespread vitamin D deficiency among Bangladeshi women and all of the study subjects except one have vitamin D deficiency according to the current definition irrespective of RPL or without RPL outcome. This can be attributed to the traditional clothing with whole body coverage, dark skin, disinclination of the women to have mid-day sun exposure and low amount of vitamin D containing food uptake. Lower level of vitamin D3 was also observed among Bangladeshi population in several previous studies (36-38). Our results are in agreement with several previous reports where vitamin D deficiency were reported to be associated with pregnancy losses (13,14,23-25,39). Recently, Ghaedi *et al*. also observed low serum vitamin D levels (<8 ng/ml) among RPL patients in Iran (39). Consistently, lower level serum vitamin D3 was found in Danish women with miscarriage between 10 and 22 week of gestation (26). In addition, it was observed that endometrial cells in women with recurrent spontaneous abortion were deficient in production of or response to vitamin D (12). In contrast, no significant difference of serum 25(OH)D3 concentrations was found in gestational weeks 10–14 between two groups in an Australian cohort (40). Widespread speculation that 25(OH)D has a protective role against the pregnancy loss due to its involvement in the regulator of innate and adaptive immune functions (41). A recent study demonstrated that vitamin D receptor is expressed in several immune cells (42) and is involved in a pathway controlling antigen receptor signaling and T-cell activation (43). Biologically active form of vitamin D3 (1,25(OH)2D3) has been shown to downregulate Th1 cytokines, inhibit the secretion of proinflammatory cytokines such as TNF-a, IL-6, and interferon-γ in the placenta (44-46). Accordingly, it is presumed that vitamin D in human placenta might have a direct role on the cytokine profile and the inflammatory response during gestation period (46). Recently, it has been shown that women with reduced level of 25(OH)D3 had increased risk of autoimmune and abnormal cellular immune responses (23). Accordingly, it has been reported that stimulation with 1,25(OH)2D3 modulated the release of cytokines in endometrial cells of women with spontaneous recurrent miscarriage in vitro (12). Taking all the facts together, it is plausible to hypothesize that biologically active form of vitamin D has a protective role at the feto-maternal immunologic interface and subsequently in pregnancy loss. The major strength of our study is the prospective design, where women were recruited from the general population who intended for pregnancy and did not know their vitamin D concentrations and the season of sampling which was restricted to summer only.

The participants were randomly recruited from the background population of 22- to 33-year old women living in the community. After the study, we now have tentative knowledge of serum 25(OH) D3 level in the general population of young Bangladeshi women, which can be considered as representative value at the time of pregnancy loss. Although our study comprises lower sample size, we were able to find an association between low 25(OH)D3 and pregnancy loss in a homogenous population. The limitation of the study was its comparatively smaller sample size. Additionally, the lack of validated methods to estimate the dietary vitamin D intake and the measurement of serum vitamin D concentration such as, LC-MS/MS. It was not possible to measure the various epimers of vitamin D3 during immune assay based detection. We were also unable to measure the biologically active form of vitamin D3 (1,25(OH)2D3) due to unavailability of appropriate method. Nevertheless, we do not anticipate that the Bangladeshi women differ prominently in terms of mean dietary vitamin D intake and sun exposure. In this study, blood samples were collected according to standard procedures and analyzed blindly, which has minimized the risk of information bias and pre-analytic variation. In our study, pregnancy was confirmed by ultrasound approach. Although major pregnancy losses were recorded in our study based on the participant's clinical history, potential early miscarriages might have been missed.

Conclusion

In conclusion, we found that reduced serum vitamin D concentration and intake of vitamin D containing food have an association with RPL outcome. Further studies with larger sample size and randomized controlled trials should be carried out to validate our findings.

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Conflict of interest: We state that there is no conflict of interest in this work exists in this manuscript.

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