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Association Of 25-Hydroxyvitamin D With Antioxidant Vitamins, Calcium, C-Reactive Protein And Nutritional Status In Children From Rural Setting Of Odisha, India

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ABSTRACT:

Vitamin D deficiency (VDD) is an emerging public health problem in all the age groups across the country. Many factors such as malnutrition, deficiency of vitamins, minerals and inflammation in the body are probably involved in VDD. This study was aimed at assessing the relationship of serum vitamin D (25-dihydroxyvitamin D) with antioxidant vitamins (A and E), calcium, anthropometric indicators and inflammation among 40 children aged 1-12 years (boys 24 and girls 16). Nutritional status was assessed by anthropometric z-scores. Plasma vitamins A (retinol), vitamin D and vitamin E (α–tocopherol), calcium and C-reactive protein (CRP) concentrations were estimated by standard procedures and their deficiency was reported using defined cut-off values. Mean (±SD) vitamin D level was 24.4±8.06 ng/ml, and 25% of children had VDD (<20 ng/ml). Girls were found to have significantly lower vitamin D levels than boys (21.0±8.43 vs. 26.6±7.32, p=0.033). Calcium was positively correlated with Vitamin D (r=0.385, p=0.014) and its level was found to be lower in children deficient of vitamin D. Subclinical VDD was associated with thinness of children (OR=4.3, CI=0.84-22.5, p=0.068). An inverse relationship was seen with inflammatory indicator: CRP levels with subclinical VDD [p=0.039, OR=10.92 (CI 0.55-217.60)]. The percentage of children deficient in calcium, vitamin A and vitamin E were found to be high in those having VDD. Therefore it is suggested that supplementation/food fortification of vitamin D in children may help to improve calcium status as well as their nutritional status. Moreover, further studies are needed for assessing VDD and its association with other vitamins and minerals so as to plan for preventing combinational nutritional deficiencies in vulnerable sections of population.

Keywords: vitamin D, vitamin D deficiency, calcium, malnutrition, children, Odisha, India;

Introduction:

Vitamin D (VD) is a group of fat-soluble micronutrients responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate and zinc, playing a vital role in bone health via regulation of calcium metabolism [1]. Although VD is obtained to some extend from a limited number of foods and dietary supplements, the body produces VD primarily when the skin is exposed to ultraviolet sunlight. If the supply of VD is restricted, bone becomes the main target organ for calcium release, which leads to increased bone turnover and risk of bone loss in the long term. It is well established that prolonged and severe vitamin D deficiency (VDD) leading to rickets, the childhood form of osteomalacia. Less severe form of VDD is referred to as mild, marginal or vitamin D insufficiency which may increase bone turnover [2, 3]. Both forms of vitamin D called ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) are converted to 25-hydroxy-vitamin D [25(OH)D] in the liver, and the serum/plasma level of 25(OH)D is measured to determine the adequacy of vitamin D status [4]. Normally, D_3 accounts for approximately 95% of the total circulating 25OH-D pool, whereas D_2 represents a minor fraction. More severe deficiency in 25(OH)D leads to clinical myopathy, osteomalacia in adults and rickets in children [5]. The detrimental effects of insufficient vitamin D before the appearance of skeletal deformities led to a growing interest of VDD and diagnosing the pre-rachitic and subclinical VDD is important for non-skeletal health benefits [6].

When circulating 25OH-D concentrations are inadequate (hypovitaminosis D), intestinal calcium absorption and bone mineralization are impaired. Calcium

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(Ca) is one of the most abundant trace elements present in the body shown to activate the enzymes involved in the production of reactive oxygen species and free radicals by the mitochondria [7]. Besides the effects in calcium and bone metabolism, epidemiological studies have shown anti-inflammatory and immune-modulating properties of VD [8].

Although vitamin A is essential in vision, immune response and epithelial cell growth and repair, excessive administration of vitamin A may interfere with absorption and/or metabolism of vitamin D, calcium and phosphorous [9]. Vitamin E is also known as a potent antioxidant essential for all cellular membrane functions to maintain the immune system [10]. Vitamin E deficiency (VED) may lead to rickets development, probably mediated to a certain extent through its influence on metabolism and functions of vitamin D [11].

VDD during pregnancy leads to secondary hyperparathyroidism and hypocalcaemia in the neonates. Vitamin D requirements vary based on customary calcium intake during pregnancy and lactation [12]. Although stunting and underweight are frequent and probably associated with rickets [13], association of anthropometric indicators with subclinical VDD is yet to be established. Many countries implemented vitamin D food fortification policy for prevention of rickets; however, no such programme exists in India. Though majority of population in India are in areas receiving ample sunlight throughout the year, VDD is very common in all the age groups [14]. Reports on the status of subclinical vitamin D and calcium in India are meager and almost none from Odisha. Therefore the present study aims to determine the subclinical status of vitamin D and its relationship with vitamin A, vitamin E, calcium and malnutrition in children from rural setting in Odisha, India.

Methods

The study conducted during 2012-2013 included 40 children aged 1-12years (boys 24 and girls 16) free of illness and some chronic diseases from six rural villages of Bhubaneswar, Odisha. Informed written consent was obtained from the parents of each child after the study objective was explained. Confirmation of a child's age was made with the mother along with the help of Anganwadi workers, community health workers and a local-events calendar. The Institutional Human Ethical Committee approved this study.

The anthropometric measurements were taken in duplicate and the average compounded following international recommendations. Weight of the children was obtained by a lever-based weighing machine (Seca, Germany) to nearest 100 gm and height to the nearest millimeter by an anthropometric rod, with the participant wearing standardized clothing and no shoes. Body mass index (BMI) for each child was calculated based on the ratio of weight (kg) to height (meters^2). Height and BMI data were transformed to *z*-scores, namely height-for-age (HAZ) and BMI-for-age (BAZ) using the WHO Growth Standards [15]. *Z*-scores allow comparison of an individual's height or BMI, adjusting for age and sex relative to a reference population, expressed in standard deviations (SD) from the reference mean. Children with <-2SD of HAZ were indicated as stunted while those with <-2SD of BAZ were indicated as thinned [16].

Two milliliter of blood sample was collected from each child in EDTA-containing evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) and a subsample was also collected in vials without EDTA with the use of disposable single-use needles (Dispovan, India). The plasma was separated and stored at -20^{0}C till analysis. 25-Hydroxy vitamin D levels were measured using a commercial 25-OH vitamin D_3 Elisa kit (Immunodiagonistic System Limited, UK). C-reactive protein (CRP) was estimated from the blood samples of 28 children using Elisa (DRG® CRP, DRG International Inc., USA). Plasma levels of retinol (primary form of vitamin A) and α-tocopherol (primary form of vitamin E) were measured simultaneously with high-performance liquid chromatography system (Shimadzu LC-20A) following the method of Tee and Khor [17].

The whole blood samples without EDTA were lyophilized to make powder form using freeze-dried Centrivap concentrator (Labconco make no 010112731E). The dried blood was ground to powder and mixed with high purity graphite powder in 1:1 ratio by mass and pellets were

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made by applying 6 Dalton pressure in a hydraulic press. The calcium concentration (ppm) was estimated using the proton-induced x-ray emission (PIXE) analytical method at the Institute of Physics, Bhubaneswar. Spectra were recorded using a multichannel analyser calibrated with ^{241}Am x-ray source. No X-ray absorbers were used between the detector and target during data collection. The PIXE spectral analyses were performed using GUPIX-2004 software (University of Guelph, Guelph, Ontario, Canada). The accuracy of the method was verified analyzing an International Atomic Energy Agency (IAEA) A-13 blood. For purposes of the analysis, hypovitaminosis D is defined as 25OH-D concentrations below 20 ng/mL [18] and calcium levels below 220 ppm as hypocalcaemia [19]. Retinol concentrations <20 µg/dl was characterized as being VAD [20] and α-tocopherol <0.5 mg/dl as VED [21]. CRP concentration was considered elevated above 10 µg/ml [22].

The statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) for Window version 16, Chicago, USA and Microsoft Excel. The continuous variables are expressed as mean and standard deviation (SD) or classification variables as proportions. The statistical difference between groups was calculated by the unpaired Student's t-test. The relationships between covariates were determined with Pearson's correlation coefficients. Association of each of the categorical variables with VDD (outcome variable) was computed with the Chi-square test and the strength of their association is measured by unadjusted odds ratio (OR) and 95% confidence interval (CI). Variables showing statistically significant association with the outcome variables were considered as potential risk factors for VDD. A p-value of <0.05 was considered significant.

Results

Table 1: Subclinical vitamin D [25(OH)D (ng/mL)] status among child population by age and sex

Age group	Sex	N	Mean±SD	p-value	Normal (≥20)	Deficient (<20)	p-value	OR (95% CI)
Preschool children (1-5 years)	Total	16	26.0±7.87	0.471	62.5(10)	37.5(6)	0.242	3.824 (0.15-94.20)
	Boys	14	26.6±8.29		57.1(8)	42.9(6)		
	Girls	2	22.1±0.14		100.0(2)	0.0(0)		
School children (6-12 years)	Total	24	23.3±7.99	0.084	83.3(20)	16.7(4)	0.064	9.000 (0.43-189.10)
	Boys	10	26.6±5.64		100.0(10)	0.0(0)		
	Girls	14	20.9±8.74		71.4 (10)	28.6 (4)		
Total (1-12 years)	Total	40	24.4±8.06	0.033*	75.0 (30)	25.0 (10)	1.000	1.000 (0.23-4.31)
	Boys	24	26.6±7.32		75.0 (18)	25.0 (6)		
	Girls	16	21.0±8.43		75.0 (12)	25.0 (4)		

OR = Odds ratio, CI = Confidence interval

Table 1 represents subclinical vitamin D status among rural children by age and sex. The overall vitamin D level in the study population was 24.4±8.06, and girls found have significantly lower concentrations than boys (26.6±7.32 vs. 21.0±8.43, p=0.033). School-age children had relatively lower vitamin D level as compared to preschool children (23.3±7.99 vs. 26.0±7.87). Overall, prevalence of subclinical VDD was 25%. School-age girls had significantly lower levels of vitamin D than their male counterparts in terms of mean level (26.6±5.64 vs. 20.9±8.74) and percentage of deficient (0.0% vs. 28.6%) having borderline of significances (p=0.084 and 0.064 respectively).

Table 2: Biochemical and anthropometric measurements in children in relation to sub clinical vitamin D status (Mean±SD)

Study variables	Total	Subclinical Vitamin D status [25(OH) D in ng/ml]		p-value
		Normal ≥20	Deficient (<20)	
Height-for-age (z-score)	-1.2±1.12	-1.2±1.01	-1.1±1.47	0.746
BMI-for-age (z-score)	-0.8±1.18	-0.6±1.20	-1.4±1.02	0.106
Calcium (ppm)	352.9±106.40	370.5±102.10	299.9±106.20	0.068
C-reactive protein(µg/ml)	5.9±7.47	7.4±8.77	3.3±3.20	0.172
Retinol (µg/dl)	43.2±31.89	43.4±31.85	42.4±33.75	0.929
α-tocopherol(mg/dl)	0.6±0.52	0.7±0.52	0.6±0.52	0.564

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Table 2 depicts association subclinical vitamin D status with anthropometric and biochemical measurements in study children. When the parameters were compared between children having normal vitamin D level (≥20 ng/mL) with deficient (<20 ng/mL), no significant differences was observed, however, the mean calcium was found to be lower in vitamin D deficient children as compared to children having normal level of vitamin D (370.5±102.1 vs. 299.9±106.2) which had a border line of significance (p=0.068).

The comparison of different micronutrient deficiencies according to prevalence of subclinical vitamin D deficiency in children is shown in Table 3.

Table 3: Risk factors associated with subclinical vitamin D deficiency among children

Study variable	Total	Vitamin D (25OHD) ng/mL		OR (95% CI)	p-value
		Normal(≥20.0)	Deficient(<20)		
Height-for-age z-score					
Normal (>-2SD)	72.5 (29)	73.3 (22)	70.0 (7)	1.18 (0.24 - 5.70)	0.838
Stunting (<-2SD)	27.5 (11)	26.7 (8)	30.0 (3)		
BMI-for-age z-score					
Normal (>-2SD)	80.0 (32)	86.7 (26)	60.0 (6)	4.33 (0.84-22.48)	0.068
Thinness (<-2SD)	20.0 (8)	13.3 (4)	40.0 (4)		
Blood calcium (ppm)					
Normal >220	85.0 (34)	86.7 (26)	80.0 (8)	1.63 (0.25-10.58)	0.609
Deficiency <220	15.0 (6)	13.3 (4)	20.0 (2)		
Vitamin A (µg/dl)					
Normal >20	75.0 (30)	80.0 (24)	60.0 (6)	2.66 (0.57-12.56)	0.206
Deficiency ≤ 20	25.0 (10)	20.0 (6)	40.0 (4)		
Vitamin E deficiency (mg/dl)					
Normal ≥0.5	55.0 (22)	60.0 (18)	40.0 (4)	2.25 (0.53-9.70)	0.271
Deficiency < 0.5	45.0 (18)	40.0 (12)	60.0 (6)		
C-reactive protein (µg/ml)					
Normal <10	78.6 (22)	66.7 (12)	100 (10)	10.92 (0.55-217.6)	0.039
Inflammation≥10	21.4 (6)	33.3 (6)	0.0 (0)		

BMI=Body mass index, OR=Odds ratio, CI=Confidence interval

The bivariate analysis showed higher prevalence of thinness (BMI-for-age z-score <-2SD) among children with subclinical VDD than normal with marginal significance [p=0.068, OR=4.33 (CI 0.84-22.48)]. However, an inverse relationship was observed between inflammation and subclinical VDD [p=0.039, OR=10.92 (CI 0.55-217.60)]. Blood calcium deficiency in the sample population found to be 15%. Although the percentage of calcium, vitamin A and vitamin E deficient were found to be high among VDD, however, it did not show any statistical significance. Among study variables, only calcium was significantly correlated with vitamin D (r=0.385, p=0.014) (Table 4).

Table 4: Pearson's correlation between Vitamin D and anthropometric/biochemical indicators in children.

Parameters	N	r value	p value
Height for age z score	40	-0.037	0.822
BMI for age z score	40	0.011	0.947
Calcium	40	0.385	0.014
C-reactive protein	28	0.282	0.145
Retinol	40	-0.241	0.305
α- Tocopherol	40	0.096	0.552
BMI = Body mass index, r value = Pearson's correlation coefficient			

Discussion

The present study reveals that 25% of children had subclinical VDD. School children had a lower level of vitamin D than that of preschool children. As the age from 6-12 years is the age for growth and skeletal structure development and age of 11-12 year represents first signs of

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adolescence, school children need more nutritional supplements. Children, particularly infants, may require less sun exposure to produce sufficient quantities of vitamin D because of greater capacity to produce than older people [23]. Data from national surveys show that the prevalence of low vitamin D status is less of a concern for children than for adolescents [24, 25]. It is surprising and disturbing to note that hypovitaminosis D is highly prevalent even in tropical countries like India with adequate sunshine [26]. Modern lifestyle changes have significantly reduced the total duration of sun exposure in children. UV-B, having shorter wavelength, tend to scatter earlier or later part of the day length and hence cutaneous vitamin D synthesis is maximum between 10 AM to 3 PM, the time when most of the children are either in school or indoors [27]. It was shown that in adolescent from Northern Ireland, even during summer and autumn, when serum $25(OH)D_3$ levels should be at their highest, about 9–15% of 11-15year old boys and girls had VDD [28]. It may be due to dietary insufficiency of vitamin D than the recommended level in many children and especially in many adolescents [29]. In another study in India in healthy adolescents of Delhi, 25-hydroxyvitamin D levels were below the cut-off value in 27% and 42% of children of high and low socioeconomic status, respectively and boys had higher levels than girls [30]. In conformity with the above observation, our findings show a significantly lower level of vitamin D among girls with borderline of significance in schoolgirls having 28.6% of VDD. Outila *et al.*[31] found that 13.5% and 62% of 14–16 year old girls had severe and marginal VDD, respectively in Finland. Moore *et al.*[29] estimated 50% and 32% of girls aged 9–13 years and 14–18 years, respectively, are meeting the dietary reference intake for vitamin D. Suboptimal intakes of vitamin D in adolescent girls have been well established [32]. Deficiency of 25-hydroxyvitamin D levels (<20 nmol/l) is reported in 963 school children in Tehran, which is five times more prevalent in girls than in boys [33].

In our study, calcium level was found to be low in VDD children as compared to those non-deficient (299.9±106.2 vs. 370.5±102.1), as well as it was found to be significantly positively correlated with vitamin D ($r = 0.385$, $p = 0.014$). It has been reported that low vitamin D levels were associated with low calcium intake as well as limited sunlight exposure in Chinese and Mongolian populations [34]. Calcium deficiency was observed in 15% of children in our study. Prevalence of rickets was shown to be common in children mostly attributed to severe mixed calcium and VDD rather than isolated VDD [35]. It was previously established that the principal function of $1,25(OH)_2D_3$ is to maintain serum calcium and phosphate concentration within the physiologically acceptable range for the normal mineralisation of bone [36]. Moreover, low calcium intake and high fibre diet may deplete vitamin D stores [37]. While prolonged and severe VDD leads to rickets in children, it is possible that a more marginal deficiency of vitamin D during early life may contribute to osteoporosis risk as well as potentially to the development of various other chronic diseases which are frequent in Western societies [2].

Studies have shown that fat and lean mass were not independently associated with vitamin D status in healthy-weight sample [38]. However, we found percentage of VDD was marginally associated with thinness of children ($p=0.068$). Further, rickets is shown to be common in malnourished children [39]. VDD was related to slower linear growth in girls [40]. In Saudi Arabia, BMI was reported to be significantly lower in females with VDD [41]. In contrast many studies showed inverse relation between vitamin D status and BMI-for-age [42] and obesity found to be associated with VDD in children [25].

In our earlier report it was found that both vitamin A and vitamin E contribute to imunoregulatory functions, since the group of children deficient with vitamin E had high lymphocyte count , which was even still higher when children with VAD added to VED [43]. It was also reported that vitamin D has an immunomodulation property [44]. There is an antagonistic interaction between vitamins A and D [9] as well as an inverse correlation with vitamin E [45]. Vitamin A reduces the toxic effects of vitamin D and it was also seen that vitamin D synergists with vitamin E [46].When vitamin A doses are high it antagonizes the rapid intestinal calcium response to physiological levels of vitamin D [47]. Excessive storage of vitamin A by liver and kidney may interfere with the metabolism of vitamin D to its active or inactive forms, as well as it further interferes with the intestinal absorption of vitamin E resulting synergism with vitamin D [9]. In our study no such type of interaction was found between level of vitamin D with A & E or of VDD with VAD & VED indicating that the level of vitamin A in this sample is not present in over amount (range: 3.48-92.8µg/dL) thereby unable to show its antagonistic effect

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with vitamin D or vitamin E. However, children with VAD and VED were found to be more among children with VDD.

In this study level of CRP, the biomarker of inflammation was found to be significantly associated with vitamin D. Children having normal vitamin D levels also had elevated CRP level (>10 µg/ml). It has also been reported previously that 25(OH)D at a level ≥21 ng/ml is associated with an increase in serum CRP and it is possible that the role of vitamin D supplementation to reduce inflammation is beneficial only among those with a lower serum 25OH-D [48]. The limitation of this study is small sample size that included 10 children of VDD and further study is required to confirm the results covering adequate sample. However, literature shows no relationship between inflammatory markers and vitamin D_3 levels [49].

Risk factors for developing VDD include low maternal levels of vitamin D, indoor confinement during the day, living at higher altitudes, living in urban areas with tall buildings, air pollution, darker skin pigmentation, use of sunscreen and covering much or all of the body when outside [50]. Vitamin D deficiency is not merely concomitant with calcium deficiency but also associated with several other parameters. In children supplementation or food fortification of vitamin D may improve calcium status as well as their nutritional status. No association was found between subclinical VDD and stunting (chronic undernutrition) while marginally with thinness. As the level of vitamin D is associated positively with CRP level, this should be confirmed with adequate sample of VDD so as to establish the relationship. More studies are needed on the interaction of fat-soluble vitamins A, D and E since their inter-metabolic pathways are not yet well established. As vitamin A prophylaxis programme is on-going for preschool children in India, keeping in view of public health significance, a screening programme is needed to assess the effect of vitamin A supplementation on the level of vitamin D and their deficiencies among at-risk population groups and at times of infectious outbreaks in formulating strategies that may include multiple vitamin supplementation so as to combat rickets and malnutrition.

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