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Impact of three different daily doses of vitamin D3 supplementation in healthy school children and adolescents from North India: A single-blind prospective randomized clinical trial

Running Title: Vitamin D supplementation in school children

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Abstract:

The information about adequate daily dose of vitamin D3 supplementation in school children is lacking from India. Hence, we undertook this study to evaluate the adequacy and efficacy of different doses of vitamin D3 in school children. One thousand eight vitamin D deficient (VDD) children, aged 6-16 years with serum 25OHD levels <20 ng/ml, were cluster randomised into three groups (A-344, B-341, C-232) for supplementation (600IU, 1000IU and 2000IU daily) of vitamin D3 under supervision for 6 months. Of 1008 subjects who completed the study, 938 (93%) were compliant. Baseline and post-supplementation fasting blood and urine samples were evaluated for calcium, phosphates, alkaline phosphatase, 25OHD and parathormone and urine calcium-creatinine ratio. The mean age of the subjects was 11.7±2.4 years and overall mean baseline serum 25OHD level was 9.7±3.8 ng/ml. Post-supplementation rise of serum 25OHD in compliant group was maximum with 2000 IU (28.0±12.0ng/ml), followed by 1000 IU (18.7±9.0ng/ml) and 600 IU (14.6±7.4ng/ml) and serum 25OHD levels of ≥20 ng/ml were achieved in 71.5%, 81.8% and 92.9% in group A to C respectively**.** Secondary hyperparathyroidism decreased from 31.7% to 8.4% post-supplementation. Two participants developed hypercalciuria but none developed hypercalcemia. Children with VDD benefit maximum with the daily supplementation of 2000 IU of vitamin D3. Whether recommendations of 400 IU/day by Indian Council of Medical Research or 600 IU by Indian Academy of Pediatrics or Institute of Medicine would suffice to achieve vitamin D sufficiency in children with VDD remains debatable.

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Keywords: Vitamin D3 supplementation; Vitamin D deficiency; Secondary hyperparathyroidism; Children and Adolescents

Introduction:

Vitamin D is an important micronutrient required for not only maintaining of calcium balance and safeguarding skeletal integrity but also for overall health and well-being of all age groups[1]. Presently, vitamin D deficiency (VDD) is recognised as a global epidemic[2,3]. Despite, adequate sun-shine throughout the year, VDD has been reported among all age groups and both genders from different parts of India[4-9]. This has been primarily attributed to poor sun exposure due to cultural avoidance of skin exposure, crowded houses with limited sun exposure, work culture of staying indoors, dark skin complexion, atmospheric pollution, vegetarian foods habits, absence of food fortification with vitamin D, and poor intake of vitamin D supplements[4,10]. Though vitamin D is synthesized in the skin on exposure to ultra violet radiation, vitamin D sufficiency is difficult to achieve in all seasons solely through sun exposure in children as observed in two of our studies[11,12]. Fortification of widely consumed staple foods offers a simple, practical, effective and safe alternative for combating VDD and is being practiced all over world[13]. The food fortification program in India is still in the stage of infancy[4,14]. Food Safety and Standard Authority of India (FSSAI) under section 16(5) of Food Safety and Standards Act (2006) relating to standards for food fortification has permitted voluntary fortification of milk and oil with vitamin A & D vide their letter dated 19th May 2017. Our own study in Indian school children clearly showed that providing milk fortified with vitamin D is an effective and safe strategy to deal with public health issue[15]. Although, there are several studies in literature evaluating the impact of vitamin D3 supplementation in adults[16], studies in children

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and adolescents are limited[17-25] particularly from India[26]. Duration of the available studies in children varied from 8 weeks[19,24] to one year[21,26], with supplemental doses ranging from 200IU[21,23] to 60000IU[26] administered either daily[17,19-24], weekly[19,25], bimonthly[18], monthly[18,26] or once in two months[26]. Supplementation with lower doses of 200-600 IU/day did not achieve vitamin D sufficiency in majority of VDD subjects[15,16,27]**.** Indian Council of Medical Research (ICMR) recommends daily allowance of 400IU for Indian children and adolescents[28] in contrast to 600IU/day recommended by Indian Academy of Pediatrics (IAP)[29] and Institute of Medicine (IOM)[30]. In the absence of information with regard to adequate daily dose of vitamin D3 required for Indian children with VDD[4], we undertook this study with primary aim to evaluate the adequacy and efficacy of daily supplementation of 600IU, 1000IU and 2000IU of vitamin D3 on serum 25-hydroxy-vitamin-D (25OHD) and parathyroid hormone (PTH) levels in school children and adolescents with VDD.

Material and Methods:

Subjects:

This randomized study was performed between July 2015 and December 2017. Two schools underwent supplementation in the year 2016 and the other two in year 2017. One thousand one hundred twelve school children, aged 6-16 years, who responded to our request to participate, were recruited from 4 fee paying schools in Delhi (Latitude North 28.38º, East 77.12º), India, representing mid socio-economic strata. The consent from school management, parents/guardians and verbal assent from children was obtained before undertaking this study. Parents were asked to sign the consent form, after they were provided with the details of the study in the patient information sheet and interaction of the first author with the parents to clear

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their doubts. These children and adolescents had minimal interrupted sun exposure (10-30% body surface area for approximately 30 mins/day during 9AM to 4PM. The dietary intake of vitamin D3 was minimal as most commonly consumed Indian foods contain negligible amount of vitamin D[14]. However, mean dietary intake of calcium (boys: 958±566 mg/daily; Girls 796±436 mg/ daily) was adequate and met the RDA as advised by ICMR[28]. These subjects were not advised any change in life style during the study period. The details of screening and selection of subjects for the study is given in Fig-1. Children and adolescents who were either on drugs affecting bone mineral metabolism such as calcium, vitamin D, glucocorticoids, anti-tubercular or anti-epileptics or suffering from any systemic illness were excluded from the study. Forty nine children were excluded as they did not meet inclusion criteria and rest (1063) underwent baseline investigations. Fifty five children had serum 25OHD >20 ng/ml and therefore excluded from the study. The remaining 1008 were finally recruited to participate in the study.

The students were recruited from class one to nine with three sections per class. Cluster randomisation was done within each class taking each section as a cluster, using draw of lots to maintain age parity within each group. Within a class, three sections (Clusters) were allocated for interventions [daily 600 IU (A), 1000 IU (B) and 2000 IU (C) of vitamin D3] for 6 months separately. The randomly allocated interventions were neither shared with class teachers nor with the students within each class till the end of the study. Three interventions were procured as tablets of same shape and colours packed in different yellow, green and red bottles, content of which were not known to class teacher or students. The class teachers were handed over the respective allocated intervention, to be given under supervision. Investigators were aware about

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the intervention allocation to sections, though the people involved in the laboratory analysis were blinded to the intervention status. The vitamin D3 capsules were manufactured and supplied every month by USV private Ltd, Mumbai, India. The study protocol was approved by Institute Ethical committee of All India Institute of Medical Sciences, New Delhi. This trial was registered as Clinical trial registration number**:** CTRI-2017/01/007681. We did not include a placebo arm since only vitamin D deficient children were included in the current study, and it would be unethical to supplement these children with placebo.

Data Collection:

Anthropometry measurements such as height, weight and BMI were noted at baseline. Height was measured to the nearest 0.1 cm using portable wall mounted stadiometer (Holten's Stadiometer, 200 cm/78 inches, Model WS045, Narang Medical Limited, Delhi, India) with subjects standing straight with head held in the Frankfurt plane. Weight without shoes and light clothes on, was measured to the nearest 0.1 kg, using an electronic scale (EQUINOX Digital weighing machine, Model EB6171, Equinox Overseas Private Limited, New Delhi, India). Body mass index (BMI), defined as the ratio of body weight to height square, and was expressed in kg/m^2. Weight categories were defined by revised criteria by Indian Academy of Pediatrics (IAP). Participants above adult equivalent of BMI of 23 were defined as overweight and those above adult equivalent of BMI of 27 were defined as obese[31].

Blood samples were collected in the fasting state between 0800 hrs to 0900 hrs, centrifuged and serum separated into three aliquots at the study site and transported in dry ice to the laboratory. Serum calcium, phosphorus and alkaline phosphatase (ALP) were estimated within two days of collection and the other two aliquots were frozen at -20ºC for estimation of

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serum 25OHD and PTH at a later date. Serum calcium, serum phosphate and ALP were measured by commercially available kit using automated biochemistry analyser Cobasc-501 (Roche Diagnostics, Manheim, Germany). The normal range for serum total calcium for 2-12 year was 8.8-10.8 mg/dl and 8.4-10.5mg/dl for 12-18 year old children with analytical sensitivity 0.2 mg/dl, inorganic phosphorus was 3.1- 5.3 mg/dl 7-12 year old and 2.8-4.8 in 13-16 year old children with analytical sensitivity 0.3 mg/dl, and ALP was 10-<13 years 129-417 U/L, 13-<15 years 57-254 U/L, 15-<18 years 50-117 U/L for girls and 10-<13 years 129-417 U/L, 13-<15 years 116-468 U/L, 15-<18 years 82-331 U/L for boys with analytical sensitivity 5U/L. The serum 25OHD was assayed using chemiluminescence method (Diasorin, Stillwater, MN, USA) and PTH (reference range: 10-65 pg/ml, analytical sensitivity 0.7 pg/ml) using electrochemiluminiscence assay (Roche diagnostics, GMDM-Mannheim, Germany) respectively. Intra- and inter assay coefficient of variation was 3.5% and 5% for serum 25OHD and 2.4% and 3.6% for serum PTH. Serum 25OHD level of <20 ng/ ml was defined as VDD[30]. VDD was further classified as severe (25OHD <5 ng/ml), moderate (25OHD <10 ng/ml) and mild (25OHD <20 ng/ml)[32]. Urinary samples were also collected for the random urinary calcium /creatinine ratio (UCaCrR-both calcium and creatinine measured in mg)) and was performed using Cobas-C III (Roche diagnostics, GMDM-Mannheim, Germany). Both blood and urine samples were repeated 6 months after intervention. However, in the absence of established Indian standards, diagnosis of hypercalciuria was made when random UCaCrR exceeded 0.21[33].

Intervention:

Supplementation was initiated in the month of July 2016 and 2017, every day for a period of approximately 6 months, under supervision of teachers and investigating staff at the study site

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for 6 working days/week and the records were maintained. Required numbers of vitamin D capsules were provided to the parents/guardians along with a record sheet to be maintained by the parents for Sundays and planned holidays as per school calendar. For unplanned holidays, parents were advised to collect their requirement from school. Subjects were labelled as non-compliant when they either missed taking vitamin D for more than 7 days or were regularly absenting themselves from school during the period of supplementation. There were 70 participants (7.0%) who were labelled as non-compliant, but completed the study.

Sample size calculation:

We expect that 70%, 80% and 90% children would achieve a serum level of 25OHD ≥ 20 ng/dl after 6 month of supplementation with 600IU/day, 1000IU/day and 2000IU/day cholecalciferol. This was based on our earlier studies where 70% and 81% children achieved serum 25OHD of ≥20ng/ml when supplemented with daily dose of 600IU and 1000IU of vitain D3 daily for 3 months[15]. In order to detect a significant difference among the 3 groups in a 2-sided test with a 5% α error and 80% power, 74 patients per group were required. Considering 10% loss during the follow-up period, a sample size of 82 per group was considered. The increase in sample size in this study, however, was due to the fact that we had approached all children in schools to participate and we could not refuse any child's participation.

Statistical Analysis:

Analysis was performed using Stata 12.0 (College Station, TX, USA). Descriptive statistics were calculated as mean±SD and median (min-max). Difference in the means of various parameters (continuous variables) and difference in the proportions were compared among the three study groups using Analysis of Variance (ANOVA) and chi-square test for trend. The primary outcome (serum 25OHD≥20 ng/ml) and secondary outcomes such as serum

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25OHD ng/ml and serum PTH (pg/ml) were analyzed by both intention-to-treat (ITT) and per protocol (PP) method. The missing values were imputed using baseline observation carried forward technique for the ITT analysis. The differences in percentages of serum 25OHD ≥20 ng/ml across the groups were compared using regress (adjusted for age) and *svy regress* command to account for cluster randomization. The results were presented as difference (95% CI). Paired 't' test was applied to calculate significance level of various parameters pre- and post-supplementation. Serum PTH and UCaCrR were not normally distributed. These parameters were analysed with Kruskal-Wallis test followed by Wilcoxon rank sum test and Wilcoxon signed rank test was used to assess change in PTH and UCaCrR pre- and post-supplementation. Pearson's correlation was used to evaluate relation between various parameters and change in serum 25OHD and PTH levels. Multiple linear regression analysis was carried out on delta change in hormonal parameters after adjusting for age, BMI and basal 25OHD levels. A p-value <0.05 was considered statistically significant.

Results:

The baseline anthropometric and biochemical characteristics of the participants is shown in Table-1. The mean age and BMI of the children were 11.7 ±2.4 years (boys: 11.8±2.5; girls: 11.6±2.3 years) and 18.1±3.7 kg/m^2 (boys: 18.2±3.8; girls: 17.8±3.6 kg/m^2) respectively. There was no significant difference in various parameters except for age and serum calcium levels among the three study groups (Table-1). The mean age of group C was significantly higher than those in group A & B. Bony deformities (genu valgum/varum) were present in 15.1% (152) participants. A total of 87 participants (8.6%) were obese [boys: 67 (11.5%); girls: 20 (4.7%)] and 187 (18.7%) were overweight [boys: 113 (19.3%); girls 74 (17.5%)]. Number of participants

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with obesity and overweight did not differ significantly between trial groups [obesity: 9.6%, 8.8% and 7.4%; overweight: 16.3%, 19.6% and 19.8% from group A to C respectively]. There were 14.6% (147) participants with severe, 46.8% (472) with moderate, and 38.6% (389) with mild VDD.

Vitamin D status

The overall mean baseline serum 25OHD level of 9.7±3.8 ng/ml (boys: 10.5±3.9; girls: 8.7±3.5; $p<0.0001$) increased significantly to 31.1±11 ng/ml ($p<0.001$) with no appreciable difference in the post-supplementation serum 25OHD levels between boys and girls (boys: 30.2±10.3; girls 30.1±11.8 ng/ml; $p=0.842$). Overall, 84.1% (789) participants achieved serum 25OHD levels of ≥20 ng/ml (boys: 86.7%; girls: 80.6%). As shown in Table 2, In the intention-to-treat analysis, percentage of subjects achieving serum 25OHD levels ≥20 ng/ml increased significantly from group 'A' to Group' C' (71.5%, 81.8% and 92.9%, $p<0.0001$) respectively. The results did not change even after adjustment for age (71.2%, 81.4% and 93.6%). The differences (95% CI) in the percentage of subjects achieving serum levels of 25OHD ≥20 ng/dl between the supplementation groups, A vs B, A vs C and B vs C were 10.3 (4.7, 15.9), 21.4 (15.6, 27.1) and 11.1 (5.3, 16.8) respectively. After accounting for cluster randomization, the difference (95% CI) between A vs B, A vs C and B vs C were 10.3 (0.87, 19.7), 21.4 (11.7, 31.0) and 11.1 (2.2, 19.9) respectively. Similarly, the significant differences were observed between A and C and A and B except for B and C in the per protocol analysis. Those who did not achieve serum 25OHD levels ≥20 ng/ml [149 children (15.9%)] had higher BMI (18.6±3.6 vs. 17.8±3.7 kg/m^2, $p=0.016$), lower baseline serum 25OHD (8.5±3.2 vs. 9.9±3.9 ng/ml, $p<0.0001$) and higher PTH (79.3±81.3 vs. 63.9±60.5 pg/ml, $p=0.007$) when compared to those who achieved serum 25OHD >20 ng/ml.

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A significant rise in Serum 25OHD following supplementation was observed in all the three groups both in intention to treat as well as per protocol analysis. Significant incremental responses in the mean serum 25OHD and percent increase in serum 25OHD levels were observed among the three groups (A-C) (Table-3).

Increase in serum 25OHD levels was negatively correlated with age (r=-0.045, p =0.169), BMI (r=-0.091, p=0.005) and baseline 25OHD (r=-0.235, p<0.0001). Serum 25OHD increase among three groups (mean±SE) remained significant even after adjusting for age, BMI and baseline 25OHD levels ('A':14.5±0.5, 'B':18.5±0.5, 'C':28.3±0.5 ng/ml; p<0.0001). Serum 25OHD increase was significantly higher in prepubertal when compared with post-pubertal children, girls than boys and severe than mild VDD subjects (Table-4).

Serum PTH Status

The median serum baseline PTH decreased from 52.3 pg/ml (12.6-845.5 pg/ml) [boys: 49.5 pg/ml (12.6-845.5), girls: 57.3 pg/ml (16.8-764.3 pg/ml] to 39.8 pg/ml (9.8-159.7 pg/ml) [boys: 33.5 pg/ml (9.8-159.7 pg/ml), girls: 38.6 pg/ml (12.3-129.4 pg/ml] following 6 months of vitamin D3 supplementation (p<0.0001). This decrease was observed in all three groups in both Intention to Treat and Per Protocol analysis categories (Table-3). Secondary hyperparathyroidism (PTH>65 pg/mL) was seen in 31.7% (320) participants [boys: 25.6% (150), girls: 40.2% (170); p<0.0001] at baseline, decreased to 79 (8.4%) post-supplementation [boys: 7.2% (39), girls: 10.1% (40); p=0.075]. The median decrease in serum PTH was not significant but the percent decrease was significant among three groups in both protocol categories. (Table-3). Decrease in serum PTH was higher in post-pubertal adolescents when compared to per-pubertal children, girls than boys, severe than mild VDD and those with secondary hyperparathyroidism (Table-4).

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Other biochemical parameters

Though the mean serum calcium and ALP decreased while serum phosphates increased significantly post-supplementation, their values were still within normal ranges. The median UCaCrR increased from 0.022 mg/mg (0.0003-0.152) to 0.032 mg/mg (0.001-0.245) following 6 months of supplementation ($p<0.001$). The decrease in the serum calcium, ALP and increase in serum phosphates and UCaCrR post supplementation was no different among the three groups (Table-5). Even though, none of the subjects in this study developed hypercalcemia, two participants from Group 'B' developed hypercalciuria following supplementation.

Discussion

In the absence of universal food fortification with vitamin D, supplementation is an effective alternate strategy to improve serum 25OHD status in India, as it has greater specificity of intervention and permits dose adjustment. There are several studies assessing the efficacy of vitamin D3 supplementation in adults[16]. However, only limited studies are available in children[17-25]. Furthermore, there are even fewer studies assessing the adequacy and efficacy of different daily doses of vitamin D3 supplementation on the increase in serum 25OHD levels in children and adolescents with VDD[19,20,24] than those without VDD[17,22-24].

A report of an expert group from ICMR recommended 400IU/day of vitamin D daily for Indians of all age groups[28] as against 600 IU/day recommended by IAP[29] and IOM, USA[30] and several other countries[34]. There is no definite data on how much daily vitamin D is required to prevent VDD and whether recommended daily allowance of 400 IU/day or 600 IU/day will suffice to combat widely prevalent VDD in India[4]. We, therefore, undertook to supplement a large cohort of school children with different daily doses of vitamin D and evaluated their adequacy and efficacy. We chose daily supplementation dose of 600IU as it is a widely

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recommended RDA in literature, a higher dose of 1000IU as per our earlier reported prediction equation[7] and 2000IU, as the estimated daily intake of vitamin D shown to achieve serum 25OHD levels of ≥20 ng/ml in 97.5% of subjects was 2098 IU/day[35].

The dose dependant increase in serum 25OHD following daily supplementation in consistent with the reports in literature with[19,20,24] or without VDD[17,22-24] and with different time durations[17-26]. We, in one of our earlier studies evaluating the impact of supplementing milk fortified with 600 & 1000IU of vitamin D3 in school children with VDD every day for 12 weeks showed almost similar increase of 11.45 and 15.73 ng/ml respectively. Likewise, percentages of children (70% and 81%) who had achieved serum 25OHD of ≥20ng/ml with 600IU and 1000IU of vitamin D in our previous study were very similar to that observed in the present study (71.5% and 81.8%) [15]. Though, there is a 3 month difference in the duration of the two studies, a recent study reported little change in the mean serum 25OHD levels following 3 or 6 months of daily supplementation[17]. Talib et al[19] from New York (USA), who carried out a study in 183 vitamin D deficient children (mean age 16.6±2.2 years) with three doses of 50000 IU/weekly, 5000 IU/daily and 1000 IU/daily, also observed a dose dependent mean increase of 24.1, 21.0 and 6.2 ng/ml in serum 25OHD respectively.

Dong et al[24] compared 400IU and 2000IU given for 16 weeks to 49 black boys and girls aged 16.3 ± 1.4 years with VDD (baseline mean serum 25OHD 13.6 ng/ml) also did show a higher increase with 2000 IU than 400 IU/day. The mean increase in serum 25OHD of 24 ng/ml with 2000 IU dose, was almost similar to the rise in serum 25OHD in the present study. Similar observation was made by Al-Shaar et al[21] in 336 Lebanese adolescents aged 13 ± 2 years while studying the impact of low (200 IU) and high dose (2000 IU) of vitamin D supplementation. The mean baseline serum 25OHD increased from 15±7 ng/ml to 36.3±22.3 ng/ml with 2000 IU, and

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to 18.6±6.6 ng/ml with 200 IU and the percentage of vitamin D deficient Lebanese children achieving sufficiency (96%) after one year of supplementation with 2000 IU/day was same as that achieved in the present study.

Similarly, increase in serum 25OHD with increasing doses of vitamin D supplementation in vitamin D sufficient children was also noted in a recent study by Sacheck et al[17] who evaluated the impact of three doses of vitamin D3 on serum 25OHD in at-risk school children where the mean baseline serum 25OHD was 22.0±6.8 ng/ml. In addition, GAPI trial (multicentre randomized dose response trial) conducted in children aged 9-13 years with mean baseline serum 25OHD of 28 ng/ml and supplementation doses ranging from 400-4000 IU/day also showed a dose dependent increase[22].

The response to supplementation in VDD subjects in the present study as well as other studies was significantly greater in terms of rise in serum 25OHD levels[21,24] as compared to subjects with baseline vitamin D sufficiency[17,22]. The response to supplementation with 2000 IU of vitamin D3 per day in the current study (27.2±12.5 ng/ml) was similar to that reported by Al-Shaar et al[21] (24ng/ml) and Dong et al (24) (21.0 ng/ml) in VDD subjects in contrast to not very large rise (10.7ng/ml) in a recent study by Sacheck et al[17] and (15.2 ng/ml) in an earlier study by Lewis et al[22] in vitamin D sufficient subjects. The rise, however, in serum 25OHD post supplementation with 1000IU in the present study was not only significantly higher than in studies carried out with vitamin D sufficient subjects (5.8 ng/ml and 5.0 ng/ml)[17,22] but also significantly higher in studies undertaken with VDD subjects (6.2 ng/ml and 6.9 ng/ml)[19,20]. This is possibly due the fact that the baseline serum 25OHD levels in the present study subjects was markedly lower than all the studies quoted above. The other possible explanation is the differences in the BMI of subjects as the serum 25OHD response is dependent on the vitamin D

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dose per unit of weight[7]. The mean BMI of subjects in the present study was markedly lower than that reported in other studies[17,19,20,22].

The overall increase of 1.8±1.0 ng/ml in serum 25OHD per 100 IU of vitamin D supplementation in the present study was significantly greater than that of 0.7-1 ng/ml reported in literature[30]. It is well known that the increment in serum 25OHD levels after vitamin D supplementation is inversely correlated with dose per unit weight[7], baseline serum 25OHD levels[19,26], and dose and duration of the study[16], which was also observed in present study explains the higher increase in serum 25OHD per 100 IU of vitamin D supplementation. Interestingly, a study among 56 vitamin D sufficient children with mean baseline serum 25OHD 28.9 ± 7.0 ng/ml, did not show any further increase in serum 25OHD with 1000 IU supplementation for 11 weeks[23]. This finding suggests that our bodies adapt to an increase in serum 25OHD as per their requirement following supplementation with vitamin D. Other supplementation studies performed in children with VDD are not comparable as vitamin D supplementation was carried out either weekly[25], or fortnightly[18] and monthly doses[18, 26].

The results of our study showed that 2000IU/day of vitamin D were required to achieve the serum 25OHD levels of ≥20 ng/ml in 94% of participants. This observation was consistent with what was reported by Rajakumar et al who showed that 2098 IU of vitamin D/day were needed to maintain serum 25OHD levels at 20 ng/ml in 97.5% of US children[35]. A systematic review and meta-analysis from Middle East and North Africa (NENA region) also suggested that a daily dose of 1000-2000IU of vitamin D will be required to obtain serum 25OHD levels of >20 ng/ml in the majority of the paediatric population[27]. These observations raise doubts about the

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adequacy of current recommendations of 400 IU/day by ICMR or 600 IU/day by IAP and IOM for Indian children and adolescents.

The possible explanations as to why 15.9% subjects in the present study did not achieve the desired levels of ≥20 ng/ml could be higher baseline BMI, lower baseline serum 25OHD and higher baseline PTH levels in these study subjects when compared to those became vitamin D sufficient. These children may require either higher supplemental dose of vitamin D or longer duration of supplementation to respond and normalise serum 25OHD as has also been observed by several other workers[16,25,39].

A significant decrease in serum PTH levels as well as decline in the prevalence of secondary hyperparathyroidism was also reported in a study from Middle East[21] and in one of our earlier studies where the decline in secondary hyperparathyroidism was reported from 50% to 7.1% when VDD children were supplemented with 60,000 IU/month for a period of 6 months[39]. All these studies had subjects with VDD and high baseline serum PTH levels. In contrast, no significant decrease in serum PTH was recorded in studies carried out in subjects without VDD[17,19,20,23] as these studies had lower serum PTH levels when compared to present study. The fact that the mean decrease in serum PTH was not statistically significant among the three groups, suggests that decrease in serum PTH is not dose dependent as also noticed in other study[22]. Since the mean decrease in serum PTH was significantly higher in participants with severe VDD and those with secondary hyperparathyroidism; it may be hypothesized that children and adolescents in the current study truly represented vitamin D deficiency as opposed to those from west who either did not truly have vitamin D deficiency or had subclinical VDD[38]. Those

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studies were probably conducted to raise serum 25OHD levels to >30 ng/ml to derive controversial extra-skeletal benefits particularly in paediatric population[39].

Persistence of secondary hyperparathyroidism in 8.4% subjects despite serum levels of 25OHD being ≥20 ng/ml may be indicative of either persistent low dietary intake of calcium in them or inability of parathyroid gland to return to it's normal functioning within 6 months despite achieving adequate levels of serum 25OHD. This is similar to what is seen with serum TSH levels remaining suppressed for months despite patient being in remission in Grave's disease with normal T3 and T4 levels. Possibility of primary hyperparathyroidism is ruled out as none of these subjects had hypercalcemia[36].

In this study, though serum levels of calcium showed statistically significant decline following vitamin D supplementation with 600 and 1000 IU of vitamin D/day, the levels were still numerically within normal limits. Whether this decrease has any clinical relevance is questionable. Hyperparathyroidism is associated with increase in serum calcium, ALP and decrease in phosphates; hence, improvement in secondary hyperparathyroidism post-supplementation may have led to decrease in serum calcium, ALP and increase in serum phosphates. Significant decrease in serum ALP levels and increase in serum phosphates levels post-supplementation has also been observed in one of our earlier reports[26]. Some other studies have reported no change in serum calcium, phosphates and ALP levels[17,23].

The UCaCrR has shown a wide variation ranging from 0.024 to 0.44 in various geographic areas[40-47]. Two early studies from India showed a mean ratio of 0.155 and 0.299 respectively[48,49]. The median value noted in the present study was 0.022 (0.0003-0.152), which significantly increased to 0.032(0.001-0.250) post supplementation. The change in UCaCrR in children following vitamin D3 supplementation has not been studied earlier, however, there are

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conflicting reports in adults[50,51]. Though hypercalcemia and hypercalciuria always remains a possibility with vitamin D supplementation as reported by Talib et al[19] in 3 children following supplementation, there was no case of hypercalcemia and only two cases of hypercalciuria were detected in the present study. It is also known that hypercalciuria and hypercalcemia are unrelated with dose and duration of vitamin D supplementation[19,52].

The main strength of our study was a large cohort of school children undertaken for daily vitamin D supplementation and evaluation of UCaCrR to detect hypercalciuria which has been done for the first time in Indian children. We did not advise any change in life style, which can be an important confounding factor and may affect the results of vitamin D intervention. However, exposure to sun[11,12] remains an important part of management of both symptomatic and asymptomatic VDD subjects. Possible weaknesses were inability to carry out individual randomization and evaluate bone formation and resorption markers.

Conclusion:

Supplementation of vitamin D with all three daily doses of vitamin D3 (600IU, 1000IU, 2000IU) resulted in significant increase in the serum 25OHD levels in school children with VDD. Children seem to benefit maximum with the daily dose of 2000IU/day with 94% achieving serum levels of ≥20 ng/ml following supplementation. The rise in serum 25OHD was inversely proportional to age, BMI and serum 25OHD levels. Whether daily allowance of 400 IU as recommended by ICMR or 600 IU by IAP and IOM, would suffice in children and adolescents with VDD to achieve serum levels of ≥ 20 ng/ml, remains debatable. Further studies are required to be undertaken before revising the earlier proposed RDAs by ICMR, IAP and IOM.

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vitamin D3 capsules for the trial. We thank Mr D H Pai Panandiker, Chairman and Ms Rekha Sinha, CEO, international Life Sciences Institute (India) for sponsoring the project. We highly appreciate the support of the principals, parents and children for participating in this trial. We would like to put on record our appreciation for the help rendered by Ms Pamela Marwaha, Dr S K Mathur and Ms Neeru Gandhi of Society for Endocrine Health for Elderly, Adolescents and Children (SEHEAC) for their valuable contribution towards this project.

Clinical trial registration number: CTRI:2017/01/007681

Contribution to authorship

RK Marwaha, G Sethuraman - Conceptualizing the study, clinical evaluation and preparation of manuscript.

M K Garg - Designing the study, analysis of data and preparation of manuscript

Nandita Gupta - Laboratory evaluation of hormones

A Mithal - Designing the study and preparation of manuscript

Navin Dang - Biochemical evaluation of samples.

M Kalaivani – Sample size calculations and Statistical analysis

M Ashraf Ganie - Recruitment and clinical evaluation of the subjects.

Archana Narang, Preeti Arora, Annie Singh, Aditi Chadha and RK Manchanda - Execution of project including sample and data collection, supervision of supplementation in all schools, sample collection and data entry

Research funding: Nestle R & D, India.

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Figure 1: Consort Flow Diagram

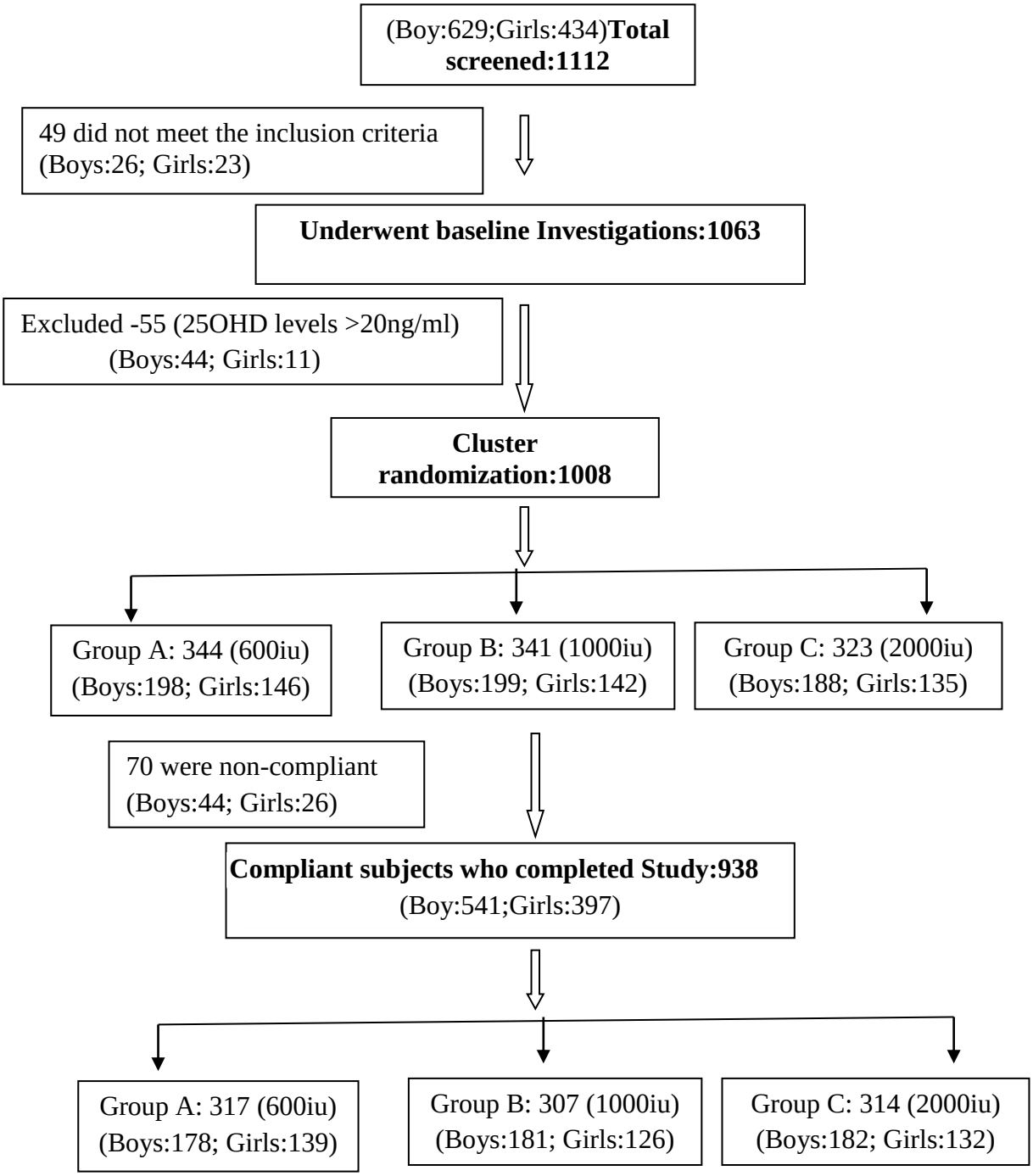

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Table 1: showing baseline demographic details and biochemical parameters

Baseline Characteristics	Vitamin D Supplementation Groups			p-value for trend
	600IU (n=344)	1000IU (n=341)	2000IU (n=323)	
Age (year)	11.5±2.4 (11.3-11.8)	11.5±2.4 (11.2-11.7)	12.1±2.4 (11.8-12.4)	0.001
BMI (kg/m^2)	18.0±3.5 (17.7-16.4)	17.9±4.0 (17.5-18.3)	18.2±3.6 (17.8-18.6)	0.531
Serum 25OHD (ng/ml)	9.6±3.8 (9.2-10.0)	9.7±3.9 (9.3-10.1)	9.8±3.8 (9.4-10.2)	0.823
Serum PTH (pg/ml)*	53.2 (12.6-764.3)	51.5 (15.0-613.4)	52.7 (16.8-845.5)	0.911
Serum calcium (mg/dl)	9.9±0.5 (9.8-9.9)	9.9±0.4 (9.8-9.9)	9.8±0.5 (9.7-9.8)	0.010
Serum phosphates (mg/dl)	4.8±0.7 (4.7-4.9)	4.8±0.6 (4.7-4.9)	4.7±0.6 (4.6-4.7)	0.041
Serum ALP (U/L)	275.3±100.1 (263.3-285.8)	274.0±109.7 (264.9-289.5)	273.4±121.6 (259.0-285.9)	0.975
UCaCrR* (mg/mg)	0.027 (0.0006-0.129)	0.022 (0.0004-0.125)	0.020 (0.0008-0.151)	0.126

* Values for serum PTH and UCaCrR are expressed as Median (range), rest is expressed as Mean±SD (95% CI)

25OHD – 25 hydroxy-vitamin D, PTH-Parathyroid hormone, ALP-Alkaline phosphatase, UCaCrR – Urinary calcium creatinine ratio

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Table 2: Comparison of percentage of serum 25OHD levels ≥20 ng/ml (primary outcome) **after vitamin D supplementation in the three groups by Intention to treat & Per protocol analysis**

Serum 25OHD ng/ml	600IU (A)	1000IU (B)	2000IU (C)	p-value
Intention to treat (n=1008)	**n=344**	**n=341**	**n=323**	
≥20 ng/ml (No, %)	246 (71.5)	279 (81.8)	300 (92.9)	<0.0001
Difference (95%CI)	Between A & B	Between A & C	Between B & C	
Unadjusted	10.3 (4.7, 15.9)	21.4 (15.6, 27.1)	11.1 (5.3, 16.8)	
P-value	<0.0001	<0.0001	<0.0001	
Adjusted for age	10.2 (4.6, 15.9)	22.4 (16.7, 28.2)	12.2 (6.5, 17.9)	
P-value	<0.0001	<0.0001	<0.0001	
Adjusted for cluster	10.3 (0.87, 19.7)	21.4 (11.7, 31.0)	11.1 (2.2, 19.9)	
P-value	<0.0001	<0.0001	<0.0001	
Per Protocol (n=938)	**n=317**	**n=307**	**n=314**	
≥20 ng/ml (No, %)	246 (77.6)	279 (90.9)	300 (95.5)	<0.0001
Difference (95%CI)	Between A & B	Between A & C	Between B & C	
Unadjusted	13.3 (8.3, 18.3)	17.9 (13.0, 22.9)	4.6 (-0.3, 9.7)	
P-value	<0.0001	<0.0001	0.067	
Adjusted for age	13.2 (8.2, 18.2)	18.4 (13.4, 23.4)	5.2 (0.1, 10.2)	
P-value	<0.0001	<0.0001	0.045	
Adjusted for cluster	13.3 (7.3, 19.2)	17.9 (10.6, 25.2)	4.6 (-0.09, 9.4)	
P-value	<0.0001	<0.0001	0.054	

25OHD – 25 hydroxy-vitamin D.

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Table-3: Comparison of mean **serum levels of Serum 25OHD and PTH in the three groups by Intention to treat & Per protocol analysis**

Outcome Measures	600IU (A)	1000IU (B)	2000IU (C)	p-value
Intention to treat (n=1008)	**n=344**	**n=341**	**n=323**	
Serum 25OHD ng/ml				
Baseline	9.6±3.8	9.7±3.9	9.8±3.8	0.838
Post-Supplementation	23.5±7.4	26.7±9.7	37.1±12.4	<0.0001
P-Value (Paired)	<0.0001	<0.0001	<0.0001	
Mean Increase	13.8 (13.0,14.7)	17.0 (15.9,18.1)	27.2 (25.9,28.6)	<0.0001
Percent Increase	183 (166.8,198.9)	229 (208.0, 250.5)	342 (315.0, 369.3)	<0.0001
Serum PTH (pg/ml)				
Baseline	53.2 (12.6-764.3)	51.5 (15.0-613.4)	52.7 (16.8-845.5)	0.911
Post-Supplementation	37.5 (12.3-126.3)	34.9 (12.2-159.7)	34.9 (9.8-109.0)	0.112
P-Value (Paired)	<0.0001	<0.0001	<0.0001	
Median decrease	15.7 (-6.1,170.4)	16.6 (-3.6, 362.9)	17.8 (-4.3, 753.6)	0.223
Percent decrease	27.6 (25.3, 29.9)	30.9 (28.5, 33.3)	31.3 (28.9, 33.8)	0.032
Per Protocol (n=938)	**n=317**	**n=307**	**n=314**	
Serum 25OHD ng/ml				
Baseline	9.7±3.8	9.6±3.9	9.8±3.9	0.796
Post-Supplementation	24.3±7.1	28.3±8.7	37.8±11.8	<0.0001
P-Value (Paired)	<0.0001	<0.0001	<0.0001	
Mean Increase	14.6 (13.8, 15.4)	18.7 (17.7, 19.8)	28.0 (26.6, 29.3)	<0.0001
Percent Increase	192 (175.3, 209.2)	251 (228.7, 272.9)	351 (323.8, 372.3)	<0.0001
Serum PTH (pg/ml)				
Baseline	52.2 (12.6-764.3)	51.5 (15.0-613.4)	52.1 (16.8-845.5)	0.934
Post-Supplementation	37.8 (12.3-126.3)	34.1 (12.3-159.7)	34.5 (9.8-109.0)	0.049
P-Value (Paired)	<0.0001	<0.0001	<0.0001	
Median decrease	15.7 (-6.1, 170.4)	16.6 (-3.6, 362.9)	17.8 (-4.3, 753.6)	0.223
Percent decrease	27.1(24.7, 29.5)	31.3 (28.8, 33.8)	31.3 (28.8, 33.8)	0.012

Serum 25OHD (ng/ml) presented as Mean±SD; Mean Increase (95% CI); Percent Increase (95% CI) and **Serum PTH (pg/ml)** presented as Median (range)

25OHD – 25 hydroxy-vitamin D, PTH – parathyroid hormone

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Table 4: Parameters affecting changes in 25-hydroxy-vitamin D (25OHD) and parathyroid hormone (PTH)

Parameters	n	25OHD increase	PTH Decrease
Age (years)			
Prepubertal (<10 years)	232	22.3±13.6 (20.5-24.0)	15.0±23.7 (12.0-18.1)
Post pubertal (>10 years)	706	19.8±10.3 (19.1-20.6)	31.0±64.3 (26.3-35.8)
P-value		0.004	<0.0001
Gender			
Boys	541	19.8±10.9 (18.8-20.7)	21.0±45.0 (17.2-24.8)
Girls	397	21.3±11.6 (20.2-22.5)	35.4±70.1 (28.4-42.3)
P-value		0.033	<0.0001
Weight (kg)			
Normal	689	20.8±11.6 (19.9-21.6)	28.8±64.1 (24.1-33.6)
Overweight	170	19.9±10.5 (18.3-21.5)	20.9±24.5 (17.2-24.6)
Obese	79	18.5±9.5 (16.4-20.6)	24.7±43.7 (14.9-34.5)
P-value		0.181	0.748
Vitamin D deficiency			
Mild	356	17.8±11.4 (16.6-19.0)	12.0±15.5 (10.4-13.7)
Moderate	444	21.1±10.8 (20.1-22.1)	26.6±52.2 (21.8-31.5)
Severe	138	25.0±10.3 (23.2-26.7)	67.2±104.4 (49.6-84.7)
P-value		<0.0001	<0.0001
Secondary hyperparathyroidism			
Present	79	14.5±6.5 (13.0-16.00	65.3±129.4 (36.3-94.3)
Absent	859	20.9±11.4 (20.2-21.7)	23.5±43.5 (20.6-26.5)
P-value		<0.0001	<0.0001

Values of increase in 25OHD and decrease in PTH are expressed as Mean±SD (95% CI)

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Table 5**: showing effect of Vitamin D supplementation on serum levels of Serum calcium, phosphates, ALP and Urinary Calcium Creatinine ratio in the three groups**

Other outcomes	600IU (A)	1000IU (B)	2000IU (C)	p-value
	n=317	**n=307**	**n=314**	
Serum Calcium (mg/dl)				
Baseline	9.9±0.5 (9.8-9.9)	9.9±0.4 (9.8-9.9)	9.8±0.5 (9.7-9.8)	0.015
Post-Supplementation	9.7±0.3 (9.7-9.8)	9.7±0.3 (9.6-9.7)	9.8±0.3 (9.7-9.8)	0.107
P-Value	<0.0001	<0.0001	0.090	
Serum Phosphates (mg/dl)				
Baseline	4.8±0.7 (4.7-4.9)	4.8±0.6 (4.7-4.9)	4.7±0.6 (4.6-4.7)	0.013
Post-Supplementation	4.9±0.6 (4.9-5.0)	4.9±0.6 (4.8-4.9)	4.8±0.6 (4.8-4.9)	0.068
P-Value	<0.0001	0.008	<0.0001	
Serum ALP (U/L)				
Baseline	274.5±101.3 (263.3-285.8)	277.2±108.7 (264.8-289.5)	272.4±119.9 (259.0-285.9)	0.867
Post-Supplementation	258.1±85.6 (248.6-267.6)	260.7±83.0 (251.3-270.0)	256.7±102.8 (245.3-268.1)	0.861
P-Value	<0.0001	<0.0001	<0.0001	
UCaCrR (mg/mg)				
Baseline	0.0261 (0.001-0.130)	0.0219 (0.003-0.125)	0.0204 (0.001-0.152)	0.108
Post-Supplementation	0.0337 (0.001-0.184)	0.0315 (0.002-0.245)	0.0345 (0.002-0.212)	0.703
P-Value	<0.0001	<0.0001	<0.0001	

* All values are presented as Mean±SD (95% CI) except UCaCrR which is presented as Median (range)

ALP=Alkaline phosphatase, UCaCrR – Urinary calcium creatinine ratio

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