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EFFECT OF VITAMIN-D3 SUPPLEMENTATION ON CALCIUM LEVELS IN FEMALES FROM PAKISTANI POPULATION

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SUMMARY

There is growing interest in the importance of vitamin-D3 not only in the maintenance of bone health but also in terms of its potential role in the prevention of non-skeletal disorders such as auto-immune diseases, cancer, mental health problems and cardiovascular diseases.

The current study was carried out to evaluate the effect of vitamin-D3 supplementation on circulating vitamin-D3 and calcium levels in post pubertal females. A total of 251 females with ages between 15 to 65 years were selected for the study. Basal serum vitamin-D3 and calcium levels were measured before supplementation (batch 0). Subjects were supplemented after 2 months (batch I), 4 months (batch II) and 6 months (batch III) and serum vitamin-D3 and calcium levels were measured after every dose. Serum vitamin-D3 and calcium levels were compared among different age groups and among different batches through ANOVA. $P<0.05$ was considered significant confidence interval.

Results showed that vitamin-D3 and calcium levels were non significantly changed in any age groups in batch 0, I and II. However, in batch III, vitamin-D3 levels in age group 56-65 were significantly higher ($p<0.041$) than those in age group 45-55 yrs. Moreover, vitamin-D3 levels were significantly elevated in all age groups after batch II that declined after batch III but remained in the normal range. The study concludes that vitamin-D3 supplementation in females with vitamin D deficiency elevated vitamin D3, as well as calcium levels, within 4 months of supplementation at 2 months interval.

Key Words: Vitamin D3, cholecalciferol, calcium, females, Pakistani

INTRODUCTION

Vitamin D is essential for strong bones and good health. It is also an important factor for muscles, heart, lungs and brain. In addition, our body can fight against infections in presence of this vitamin. Vitamin D is synthesized by the body in the presence of sunlight *(1)*. In humans, the most important compound in this group is vitamin-D3 known as cholecalciferol *(2)*.

Calcium is the most abundant mineral and constitutes about 2% of our total body weight. Almost all of this calcium is found in the skeleton. The rest is found in teeth, blood plasma, soft tissues and extracellular fluid. It is essential for maintaining balance of calcium in the blood and bone. Blood calcium is an important regulator of key bodily processes, such as muscle contraction, nerve impulse signaling and blood coagulation *(3)*.

Studies indicate that low blood serum vitaminD3 is related to breast cancer, prostate cancer, colorectal cancer, non-Hodgkin's lymphoma, multiple sclerosis, diabetes, bacterial infections, inflammatory bowel disease, elevated cholesterol, rheumatoid arthritis and common obesity. The number of premature deaths from cancer due to insufficient Vitamin-D3 in USA is up to 85,500 and up to 25,000 in UK annually *(4)*.

A diet deficient in vitamin D, in conjunction with inadequate sun exposure, causes osteomalacia that involves softening of the bones. Although, it is one of the rarest diseases in developed world vitamin D deficiency has become a worldwide issue in the elderly and remains common in children and adults *(5)*.

A correlation between hypovitaminosis D and insulin resistance has been identified in pregnant women *(6)*. Its deficiency also causes maternal ostopenia, neonatal rickets, female infertility and retard menarche age *(7)*.

Vitamin D overdose causes hypocalcaemia, anorexia and nausea. It may frequently followed by polyuria, polydipsia, weakness, insomnia, nervousness, pruritus and ultimately renal failure. Proteinuria, urinary casts, azotemia, and metastatic calcification in kidneys may also develop *(8)*.

Other symptoms of vitamin D toxicity include mental retardation in young children, abnormal bone development and growth, diarrhea, irritability, weight loss and severe depression *(9)*. The food and drug authority (FDA) has recommended tolerable upper limit for infant dose 25 µg/day (1,000 IU). In infants, doses of 1000 µg per day produced toxicity within one month *(10)*.

After being commissioned by the Canadian and American governments, the Institute of Medicine, as of 30 November 2010, increased the tolerable upper limit (UL) to 2,500 IU per day for ages 1–3 years, 3,000 IU per day for ages 4–8,and 4,000 IU per day for ages 9–71+,including pregnant or lactating women *(11)*.

Studies of vitamin D receptor (VDR) in null mice have indicated that the principal function of vitamin D in mineral homeostasis is to increase calcium absorption from the intestine. This conclusion was based on the findings that rickets and osteomalacia are prevented and serum calcium and parathyroid hormone (PTH) are normalized when VDR null mice are fed a rescue diet high in calcium and lactose *(12,13)*.

In case of low calcium levels due to insufficient dietary intake or increased body demand due to growth, pregnancy or lactation, the synthesis of the hormonally active form of vitamin D; 1,25-dihydroxyvitamin D (1,25(OH)2D3) is increased leading to enhanced intestinal calcium absorption. If normal serum calcium is unable to be maintained by intestinal absorption, then 1, 25(OH) 2D, together with para thyroid Hormone (PTH), mobilizes bone calcium and increases the reabsorption of calcium from the renal distal tubule *(12)*. The aim of the current study was to evaluate the effects of vitamin Dsupplementation on calcium levels and intrinsic levels of vitamin-D3 as measured in blood serum in different age groups of females from Pakistani origin.

MATERIALS AND METHODS

The study was randomized and prospective. It was conducted from January 2016 - June 2016 and conformed to the tenets of Declaration of Helsinki *(14)*. The study design was approved by the Ethics Committee for Research on Human Subjects, Hazara University, Mansehra, Pakistan. All the subjects were screened for any systemic disease that could bias the results. Written informed consent was obtained from all subjects after explaining the study procedure to each one of them.

The subjects were recruited from Mansehra (KPK), Rawalpindi (Punjab) and Islamabad (Capital), Pakistan. The inclusion criteria were: females, aged from 15 to 65 years. Exclusion criteria were females prior menarche, aged less than 15 years and more than 65 and suffering with diabetes, hypertension. All the subjects were divided into five age groups: Group I: 15 – 25 years, Group II: 26 – 35 Years, Group III: 36 – 45 Years, Group IV: 46 – 55 Years, Group V: 56 – 65 Years.

The first blood samples were taken before administration of vitamin-D3 and labeled as batch 0. Then vitamin D3 was

administered orally at dose of 5mg/200,000 IU (Kin-Vit-D3, Caraway Pharmaceuticals, Linear, Islamabad, Pakistan) and a second blood sample was taken after 2 months (batch I) where a second dose of vitaminD3 was administered. The third blood sample was taken after 4 months (batch II) and the third dose of vitaminD3 administered. After 6 months of last blood samples were taken (batch III).

Blood aspiration was done by a trained phlebotomist for all the subjects. Blood was taken from the cubital vein in EDTA.K3vacutainers (BD, USA). The blood was centrifuged and serum was separated. The serum samples were stored at -80 ºC until analysis.

The vitamin D3concentration was estimated in serum through ELISA, using a commercially available kit (Beckman Coulter, Czech Republic) by following the manufacturer's methods and instructions. The required number of wells were placed in the strip holder. Then, 50 µL of the calibrator, control and the samples were added to each well. Later, 150 µL of the incubation buffer was added and incubated for 2 hours at 18 – 25oC with shaking (300 to 700 rpm). Meanwhile, the HRP conjugate was prepared. After incubation, the plate was washed three times with 400µL of wash solution. Then, 200µL of HRP conjugate was added, and incubation was carried out for 30 minutes at 18–25^{o}C with shaking (300–700 rpm). After incubation, the solution was aspirated and washed thoroughly with washing solution. Then, 100 µL of tetramethylbenzidine (TMB) solution was added and incubated for 15 minutes at 18-25^{o}C (300-700 rpm). Then, 100µL of stop solution (1M HCl) was added, and absorbance was observed at 450nm through an ELISA plate reader (AMP Platos R II, Austria).

Calcium was quaintified using a commercially available kit (Analyticon, Germany). Reagent 1 (R1) was an AMP buffer (pH 10.7), reagent 2 (R2) was O-cresolphtalein complexone (0.16mmol/l), Hydroxychinoline (6.9mmol/l) and Hydrochloric acid (0.06mmol/l). Equal quantities of R1 and R2 were mixed to prepare the working reagent. The chemistry analyzer (AMP Piccos II, Polland) was used to measure calcium levels. Working reagent (2000µL) was used as the blank solution. Then, 50µL of serum (the calibrator) was added to the working reagent and incubated for 5 minutes. Then, absorbance was noted at a 590nm wavelength. Calcium concentration was calculated by the formula: calcium concentration (mg/dL) = ΔE Sample/ ΔE Calibrator × Conc. Calibrator.

Data was computed through the Statistical Package for Social Sciences (IBM, SPSS

version 20.0, Chicago, Illinois, USA). Parameters such as geographical location and profession were analyzed through non-parametric χ2analyses. Vitamin-D3 and calcium were compared in different age groups using ANOVA and post hoc analyses. The Pearson product-moment correlation coefficient (PPMCC) was applied to correlate vitamin-D3 with calcium levels. P>0.05 was considered to be the significant difference.

RESULTS

At the start of the study, serum Vitamin D3 levels were deficient (less than the threshold levels of 30-100 ng/ml) in all the subjects. The levels of vitamin D3 were not significantly different in household women as compared to those in student or working women (batch 0 p<0.125; batch I p<0.577; batch II p<0.1.05; batch III p<0.112). Calcium levels in household women were not different from students or the working women in batch 0, I and III (batch 0 p<0.946; batch I p<0.758; III p<0.743). However, calcium levels in house hold ladies were significantly greater, as compared to those in working women (p<0.021) in batch II.

Calcium levels were significantly high in subjects living in hilly areas, as compared to those living in plain areas before vitamin D3 supplementation and after batch II (p<0.0001 & p<0.007 respectively). However, the levels of vitamin D3 were not significantly different in subjects living in hilly or plain areas (batch 0 p<0.142; batch I p<0.727; batch II p<0.736; batch III p<0.569).

The number of subjects in each age group was as follows: age group 15-25 years n= 53, age group 26-35 years n= 55, age group 26-35 years n=58, age group 36-45 years n=46, and age group 56-65 years n=39. The levels of vitamin-D3and calcium were not significantly different for any age group in batch 0, batch I and batch II. In batch III, vitamin-D3 levels were significantly decreased in the age group 46-55 years, as compared to those in age group 56-65 years (p<0.041). In batch III, calcium levels were significantly reduced in both age groups 26-35 years (p<0.029) and 36-45 years (p<0.036) as compared to those in age group 55-65 years (Table 1).

Vitamin-D3 levels in age group 15-25 years were significantly higher (p<0.0001; F=32.876) in batch II, as compared to batch 0 (p<0.0001) batch I (p<0.0001) and batch III (p<0.024) (Fig. 1). Calcium levels in age group 15-25 years were significantly higher (p<0.0001; F= 12.023) in batch II, as compared to batch 0 (p<0.0001), batch I (p<0.0001) and batch IV (p<0.037) (Fig. 2). Vitamin-D3 levels in age group 26-35 years were significantly higher (p<0.0001; F=43.316) in batch II, as compared to batch

0 ($p<0.0001$) batch I ($p<0.0001$) and batch III ($p<0.001$) (Fig. 1). Calcium levels in age group 26-35 years were significantly higher ($p < 0.0001$; $F=19.624$) in batch II, as compared to batch 0 ($p < 0.0001$), batch I ($p < 0.0001$) and batch IV ($p < 0.0001$) (Fig. 2).

Vitamin-D3 levels in age group 36-45 years were significantly higher ($p < 0.0001$; $F=62.767$) in batch II, as compared to batch 0 ($p < 0.0001$) batch I ($p < 0.0001$) and batch III ($p < 0.001$) (Fig. 1). Calcium levels in age group 36-45 years were significantly higher ($p < 0.0001$; $F=28.259$) in batch II, as compared to batch 0 ($p < 0.0001$), batch I ($p < 0.0001$) and batch IV ($p < 0.0001$) (Fig. 2).

Vitamin-D3 levels in age group 46-55 years were significantly higher ($p < 0.0001$; $F=12.217$) in batch II, as compared to batch 0 ($p < 0.0001$) batch I ($p < 0.0001$) and batch III ($p < 0.02$) (Fig. 1). Calcium levels in age group 46-55 years were significantly higher ($p < 0.015$; $F=3.88$) in batch II, as compared to batch I ($p < 0.019$) (Fig. 2).

Vitamin-D3 levels in age group 56-65 years were significantly higher ($p < 0.0001$; $F=18.349$) in batch II, as compared to batch 0 ($p < 0.0001$) batch III ($p < 0.029$) (Fig. 1). Calcium levels in age group 56-65 years were significantly higher ($p < 0.001$; $F=7.015$) in batch II, as compared to batch 0 ($p < 0.001$) and batch I ($p < 0.005$) (Fig. 2).

Vitamin-D3 levels were significantly correlated to calcium levels after administration of vitamin D3; in batch I($r= 0.228$; $p < 0.036$), batch II($r= 0.260$; $p < 0.016$), batch III ($r= 0.395$; $p < 0.0001$).

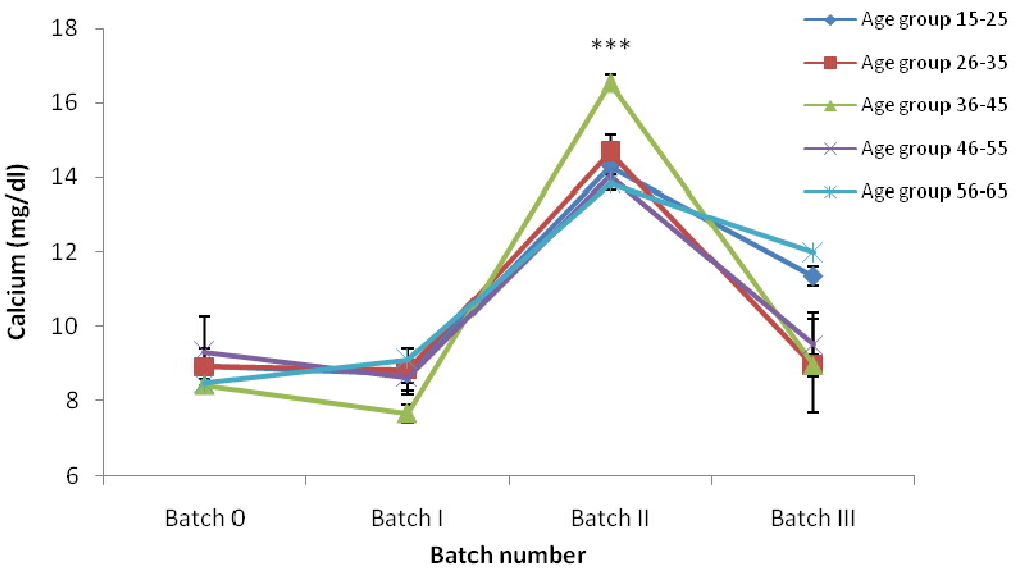
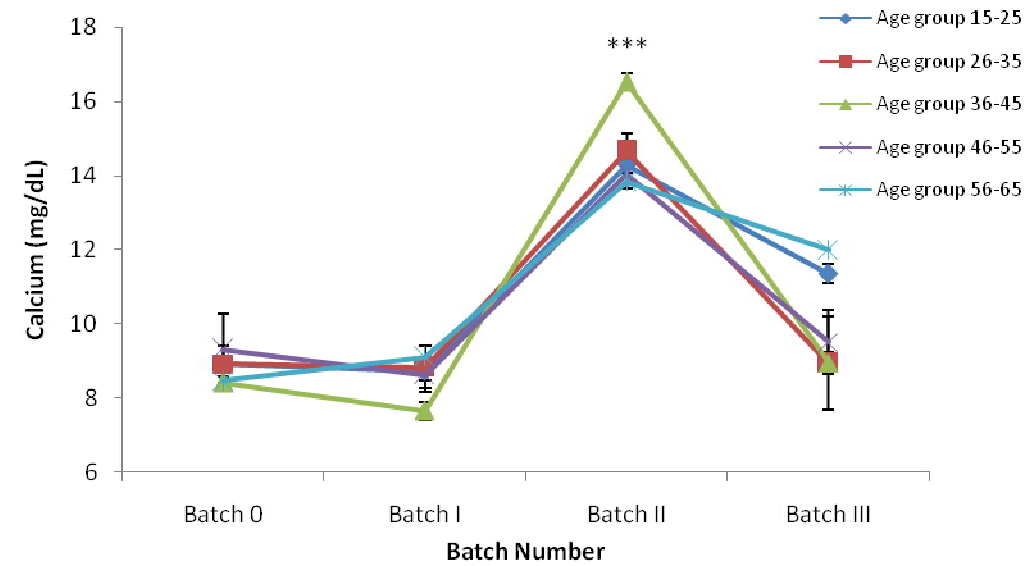


Figure 1: Comparison of vitamin D_3 levels before and after supplementation in different age groups



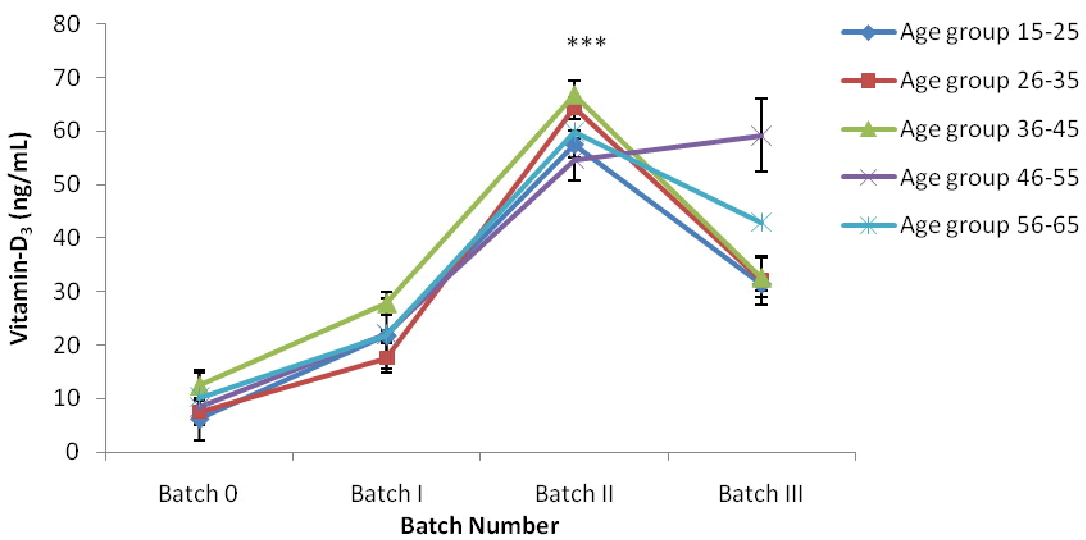


Figure 2: Comparison of calcium levels before and after supplementation in different age groups

Table 1: Comparison levels of vitamin D_3 and calcium in different age groups before and after vitamin D_3 supplementation

Age Groups (Years)	N	Batch 0		Batch I		Batch II		Batch III	
		Vitamin D_3	Calcium	Vitamin D_3	Calcium	Vitamin D_3	Calcium	Vitamin D_3	Calcium
15-25	53	6.24 ± 0.74	8.91 ± 0.17	21.76 ± 2.57	8.73 ± 0.50	57.45 ± 6.34	14.29 ± 1.01	31.41 ± 2.98	11.36 ± 0.98
26-35	55	7.54 ± 0.94	8.92 ± 0.24	17.61 ± 2.73	8.83 ± 0.57	64.25 ± 6.58	14.66 ± 1.05	32.12 ± 2.20	8.94 ± 0.47*a
36-45	58	12.44 ± 2.51	8.42 ± 0.23	27.81 ± 2.13	7.67 ± 0.48	66.74 ± 4.00	16.54 ± 1.41	32.60 ± 2.57	8.97 ± 0.38*a
46-55	46	8.39 ± 1.26	9.31 ± 0.27	22.13 ± 4.51	8.63 ± 1.26	53.69 ± 6.90	14.04 ± 1.97	29.22 ± 3.75*a	9.51 ± 0.87
56-65	39	10.14 ± 2.00	8.47 ± 0.39	21.89 ± 3.60	9.12 ± 0.34	59.83 ± 8.54	13.84 ± 1.55	42.93 ± 4.03	11.98 ± 0.95
P Value		0.068	0.135	0.136	0.544	0.628	0.612	0.045*	0.006
F Value		2.276	1.811	1.803	0.776	0.651	0.674	2.561	3.920

*a:significantly reduced from levels inage group 56-65 years; N: number of subjects
Batch 0: Pre Dose; Batch I: After 1st and 2nd Dose; Batch II: After 3rd and 4th Dose; Batch III: After 5th and 6th Dose

DISCUSSION

Optimal vitamin D levels in serum are essential to be maintained in the body for normal homeostasis. Vitamin D performs certain essential roles of the body, including gut calcium absorption, mineralization of newly formed osteoid tissue in bone, and muscle function *(15)*. The prevention of vitamin D deficiency should be a public health priority *(16)* as it may lead to certain pathologies like hypertension, heart diseases, diabetes, age-related cognitive decline, arthritis, multiple sclerosis and even cancer *(17)*.

Currently, all the subjects in the start of the study had less than sufficient levels of vitamin D. this is surprising because the amount of vitamin D depends upon the distance of locality from the equator *(18)*; hence, for a population with such an ample amount of sunshine should have sufficient levels of vitamin D. Purdah observation, sun block usage, and the fact that increased pigmentation requires a prolonged sun exposure for vitamin D synthesis may lead to this decrease in vitamin D production.

There was no significant change in vitamin D levels after administration in relation to the occupation of the subjects i.e., the levels were non-significantly changed in working and household women. It might be due to the reason that women generally do not go out; in consequence, their exposure to sunlight is limited *(19)*. However the calcium levels in the serum of working women were significantly reduced as compared to household women. This was perhaps due to elimination of calcium from the body due to their hectic routine and the physical exertion present in working women.

The current study showed that calcium levels were elevated in subjects living in hilly areas, as compared to those in plain areas; however, the vitamin D levels were not significantly different in relation to geographic location of the subjects. It has been well defined that latitude by itself is not a good indicator of potential for vitamin D synthesis or the vitamin D deficiency status of a population. Skin exposure to UV light or sunlight is the only occurrence necessary to acquire active form of vitamin D *(20)*.

The serum concentration of vitamin-D3 and calcium were significantly unchanged in all age groups before the supplementation and after batch I and II. However, in batch III, calcium and vitamin-D3 levels in the age group 55-65 years were elevated, as compared to other age groups, confirmed by a previous study done by Houghes et al., *(21)*. Vitamin D and calcium supplementation, in elderly men and women aged 65 and above, reduced chances of bone fractures by increasing calcium and vitamin D levels.

Recommended nutrient intakes are intended to prevent the development of nutrient deficiencies in virtually all healthy persons *(22)*. Vitamin D recommendations have posed a problem because, except for some fish, vitamin D is not naturally present in the food humans normally eat. Serum 25-hydroxyvitamin D *(25*(OH)D) is the objective measure of vitamin D nutritional status *(23)*.

Currently, significantly increased vitamin D and calcium levels were observed in all age groups after the second batch of vitamin D supplementation. These levels were reduced after, but remained within normal limits. Vitamin D is an important nutrient in the maintenance of bone health. The primary functions of vitamin D are the regulation of intestinal calcium absorption and the stimulation of bone resorption, leading to the maintenance of serum calcium concentration *(24)*. Currently, all of the subjects showed a deficiency of

vitamin D.In this deficiency, reduced absorption of calcium from the intestines leads to osteoclast formation. This increases the mobilization of calcium from the bones *(25)*. If vitamin D deficiency is not treated, calcium continues to be pulled from the bone, leading to rickets in children, and osteomalacia and osteoporosis in adults *(26)*.

The study concludes that the supplementation of vitamin-D3 is useful to a certain level after the onset of puberty. The supplementation affects calcium levels in the same pattern. In addition, the effect of the supplementation is correlated with age, i.e. the outcome is not as significant in aged females as it is in young ladies.

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