Serum vitamin D level, measured by two methods, in a sample of normal subjects in Basrah

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ABSTRACT

Background: **Low vitamin D levels had been reported to be common in normal subjects worldwide. Studies in the Middle East had reported extremely low levels of serum vitamin D, despite high exposure to sunlight.**

Aim: **To estimate vitamin D serum concentration in a sample of apparently healthy subjects from Basrah, by 2 methods (chemiluminescent and fluorescent assays).**

Methods: The study was carried out on apparently healthy subjects during the period from September 2018 to February 2019. Quantitative determination of the total 25-hydroxy vitamin D in serum was made using chemiluminescent microparticle immunoassay and enzyme-linked fluorescent assay. Other parameters (calcium, phosphorus, alkaline phosphatase, parathyroid hormone, hemoglobin, and erythrocyte sedimentation rate and body mass index) were also measured.

Results: **The mean level of vitamin D measured by the two methods, was 11.57±6.63 ng/ml and 13.31±6.52 ng/ml by chemiluminescent and fluorescent assays respectively. The prevalence of vitamin D deficiency was high, where more than 80% of the 57 subjects had vitamin D level below 20 ng/ml. If the cut-off point of vitamin D deficiency was taken as 10 ng/ml, around 46% of the subjects were found deficient in both methods. Although the two methods of vitamin D assay were well correlated with each other, fluorescent assay gave, on average, a significantly higher levels compared with the chemiluminescent method. Serum parathyroid hormone, showed a negative correlation with vitamin D serum levels. After excluding children and females, no significant difference was found between adult smokers and non-smokers when vitamin D was measured by both methods.**

Conclusion: **Vitamin D deficiency is common (> 80%) among normal subjects. The enzyme-linked fluorescent assay resulted in higher mean level than chemiluminescent assay. The use of a deficiency cut-off point of 10 ng/ml may be more appropriate.**

Key words: **Vitamin D, Methods of assay, Normal level**

مستوى فيتامين (د) في مصل الدم، مقاساً بطريقتين، في عينة من الاشخاص الطبيعيين في البصرة

خلفية الدراسة: تنتشر حالة نقص فيتامين (د) بشكل واسع حول العالم لدى الاشخاص الطبيعيين كما أفادت الدراسات التي أجريت في الشرق الأوسط إن مستويات فيتامين (د) منخفضة للغاية في الدم على الرغم من شدة أشعة الشمس.

الهدف: لقياس تركيز فيتامين (د) في مصل الدم (بطريقتين التلألؤ الكيمياوي والمقايسة الفلورية) في عينة من الاشخاص الاصحاء من البصرة.

الطرائق: تم اجراء هذه الدراسة على عينه من الاشخاص الاصحاء خلال الفترة من أيلول ٢٠١٨ إلى شباط ٢٠١٩. تم قياس فيتامين (د) في مصل الدم باستعمال طريقتين (المقايسة المناعية للجسيمات الدقيقة المتلألئة كيمياوياً والثانية المقايسة الفلورية المرتبطة بالأنزيم). كما تم قياس معايير اخرى (الكالسيوم، الفوسفور، إنزيم الفوسفاتاز القلوي، هرمون الغدة المجاورة للدرقية، الهيموغلوبين، مؤشر كتلة الجسم، ومعدل ترسب كريات الدم الحمراء).

النتائج: لقد كان معدل مستوى فيتامين (د) ١١.٥٧±٦.٦٣ نانوغرام/ مل و ١٣.٣١±٦.٥٢ نانوغرام/ مل (بطريقتين التلألؤ الكيمياوي والمقايسة الفلورية) كان معدل انتشار نقص فيتامين (د) مرتفعاً حيث كان لدى أكثر من ٨٠٪ منهم مستوى فيتامين (د) أقل من ٢٠ نانوغرام/ مل وتقل هذه

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النسبة الى حوالي ٤٦٪ بكلا الطريقتين عند استخدام ١٠ نانوغرام/ مل كحد فاصل لحالة نقص فيتامين (د). على الرغم من الارتباط المعتد بين الطريقتين المستعملتين لقياس فيتامين (د) الا ان المقايسة الفلورية المرتبطة بالانزيم أعطت معدل فيتامين أعلى بالمقارنة مع طريقة المقايسة المناعية للجسيمات الدقيقة المتلألئة كيمياوياً. كما ارتبط هرمون الغدة المجاورة للدرقية في مصل الدم سلباً مع مستويات فيتامين (د) في مصل الدم وبعد استبعاد الاطفال والاناث، لم نجد اختلافاً معتدّاً بين المدخنين وغير المدخنين من البالغين عند قياس فيتامين (د) بالطريقتين.

الاستنتاج: إن معدل حدوث نقص فيتامين (د) كان عالياً (أكثر من ٨٠٪). وكانت الطريقتان اللتان استعملتا لقياس فيتامين (د) مترابطتين بشكل جيد، لكن المقايسة الفلورية المرتبطة بالانزيم أعطت معدلاً أعلى من المقايسة المناعية للجسيمات الدقيقة المتلألئة كيمياوياً. كما ان استخدام ١٠ نانوغرام/ مل كحد فاصل لحالة نقص فيتامين (د) ربما يكون ملائما اكثر.

الكلمات المفتاحية: فيتامين د، طرق الفحص، المستوى الطبيعي

INTRODUCTION

Vitamin D is a lipid-soluble vitamin,[1] having two forms; vitamin D3 (cholecalciferol), and D2 (ergocalciferol). Vitamin D3 is obtained naturally after exposure to ultraviolet light type B (UVB).[2] The diet represents another source for both types of vitamin D.[3] Vitamin D has a wide range of effects on cellular functions because of the presence of its receptors in most types of cells.[4] In addition to its well-known effect on calcium and phosphate metabolism, it has other significant effects in controlling infectious diseases.[5] Calcidiol (25-hydroxyvitamin D) is the primary circulating form, and it is the most commonly measured type in serum.[6] Different cut-off points to define vitamin D deficiency were reported. Several studies had reported levels of less than 20 ng/ml (50 nmol/L) to indicate deficiency.[2, 5, 7-15] Others considered levels below 10 ng/ml (25 nmol/L) as deficient.[16-20] The prevalence of vitamin D deficiency in Saudi Arabia was reported as 98% of women and 92% of men.[21] 64% in Qatar,[9] 85% in Iran,[14] 11.3% in Jordan,[15] while in Kuwait, 81.7% of boys, 93.4% of girls were found vitamin D deficient or insufficient (< 20 ng/ml).[17] Vitamin D deficiency seems to be a world-wide problem even in western countries. Fifty five percent of hospitalized children with acute infection in Europe had serum vitamin D level below 20 ng/ml (50 nmol/L).[22] In a group of north Italian children, 46% had vitamin D deficiency and 9% had severe deficiency.[23] The extremely low levels of serum vitamin D reported by studies in the Middle East despite high exposure to sunlight and variation in vitamin D deficiency prevalence using different methods of vitamin D assay together with limited number of studies in Iraq constitute the rationale behind conducting this study which tries to estimate vitamin D serum concentration, measured by 2 methods, in a sample of apparently healthy subjects from Basrah.

PATIENTS AND METHODS

This study was carried out during the period from September 2018 to February 2019. It was conducted on 57 apparently healthy subjects (24 males and 33 females, by history and physical examination) from Basrah general population, and their consent were obtained to participate in the study. Questionnaire form was filled for each participant, which contained information regarding age, gender, educational level, occupation, sun exposure, skin color, residency, social habits (smoking, alcohol drinking), and others. In addition, body weight and height were measured to calculate body mass index (BMI < 18.5 Kg/m^2 was considered underweight, 18.5-24.9 Kg/m^2 average, 25-29.9 Kg/m^2 overweight and 30 Kg/m^2 and more obese) according to the WHO classification.[24] Peripheral venous blood samples were collected from each participant, for measurement of serum vitamin D, hemoglobin, ESR, serum calcium, serum phosphorus, serum alkaline phosphatase and serum parathyroid hormone. Vitamin D was

measured by two methods; chemiluminescent microparticle immunoassay (CMIA) using 25-OH vitamin D kit on the Architect system (Abbott Diagnostic,USA), and enzyme-linked fluorescent assay (ELFA) using VIDAS 25-OH Vitamin D total testing kits (Biomerieux, France) on VIDAS automated immunoassay platform. In the present study, the metabolically stable form of vitamin D; 25(OH)D was measured. Different cut-off points to define vitamin D deficiency were used. Serum calcium, serum phosphorus, serum alkaline phosphatase were measured by Cobas Integra 400 Plus Autoanalyzer (Roche Diagnostic, Germany), while serum parathyroid hormone was measured by Cobas E411 ECLIA Immunoanalyzer (Roche Diagnostic, Germany). Hemoglobin was measured by Sysmex hematological autoanalyzer (Japan). ESR was measured manually by Westergren method.

Statistical Package for Social Sciences (SPSS), version 20 was used for statistical analysis; *p* value < 0.05 was considered statistically significant. Paired sample t-test was used for differences between the two methods of vitamin D measurement, and Fisher's Exact test for categorical data. Pearson correlation test was performed to find out the correlation between vitamin D serum concentration and serum levels of calcium, phosphorus, alkaline phosphatase, and parathyroid hormone.

RESULTS

1. Characteristics of study participants

Fifty seven subjects were recruited for this study. Their age ranged from 9 to 71 years, with a mean of 35.2±16.0 years. Twenty four (42.1%) were males and 33(57.9%) were females. Their BMI ranged from 15.3 to 37.5 Kg/m^2 with a mean of 26.44±4.85 Kg/m^2. After excluding children and females, 8(14%) of them were smokers and 9 were non-smokers (15.8%). Fifty (87.7%) of them were practicing indoor occupations and 20 (35.1%) lived in urban areas. Those having a daily sun exposure of > 60 min were 22 (38.6%), and those with a light brown skin color were 34 (59.6%). The mean values of all measured biochemical parameters were within reported normal range except alkaline phosphatase (ALP) which showed higher mean values than normal range. The mean levels of ALP in adults (after excluding those below the age of 18 years) were 136.32±44.56 U/L (n=36) (Table-1).

Table 1. General characteristics of study participants

Characteristics		The study sample (n=57)
Age (years)	Mean ± SD	35.2 ± 16.0
	< 18	9 (15.8%)
	18-65	46 (80.7%)
	> 65	2 (3.5%)
Gender	Males	24 (42.1%)
	Females	33 (57.9%)
BMI (Kg/m^2)	Mean±SD	26.44±4.85
	< 18.5	2 (3.5%)
	18.5-24.9	27 (47.4%)
	25-29.9	15 (26.3%)
	≥ 30	13 (22.8%)
Smoking	Smoker	8 (14%)
	Non- smoker	9 (15.8%)
Skin color	White	16 (28.1%)
	Light brown	34 (59.6%)
	Dark brown	7 (12.3%)
Occupation	Indoor occupation	50 (87.7%)
	Outdoor occupation	7 (12.3%)
Residency	Urban	20 (35.1%)
	Rural	37 (64.9%)
Sun exposure	< 20 minutes/day	14 (24.6%)
	20-60 minutes/day	21 (36.8%)
	> 60 minutes/day	22 (38.6%)
Educational level	Illiterate	5 (8.8%)
	Primary	15 (26.3%)
	Secondary	18 (31.6%)
	University	19 (33.3%)
Biochemical parameters	Calcium	8.91 ± 0.84 (7.31-10.4) n = 54
	Phosphorus	3.82 ± 0.75 (2.68-6.41) n=55
	Alkaline phosphotase	163.4 ± 90.5 (74.96-440.16) n=43
	Parathyroid hormone	58.32 ± 32.83 (15.57-218.4) n=49
Hematological parameters	Hemoglobin	12.48 ± 1.59 (9.3-15.6) n=55
	ESR	33.14 ± 32.027 (5-140) n=54

2. Baseline measurement of vitamin D

The mean level of vitamin D measured by CMIA was 11.57±6.63 ng/ml, and by Enzyme-linked fluorescent assay was 13.31±6.52 ng/ml. The two methods were well correlated and the correlation was highly significant. One difference between the two methods, is that the mean of vitamin D serum level measured by chemiluminescent microparticle immunoassay method was lower than levels measured by enzyme-linked fluorescent assay (Table-2, Figure-1).

Table 2. Measurement of vitamin D [25(OH)D] in control subjects using two laboratory methods.

Methods of measurement	Serum level of vitamin D
Chemiluminescent immunoassay	11.57 ± 6.63
Enzyme-linked fluorescent assay	13.31 ± 6.52
P value	0.004

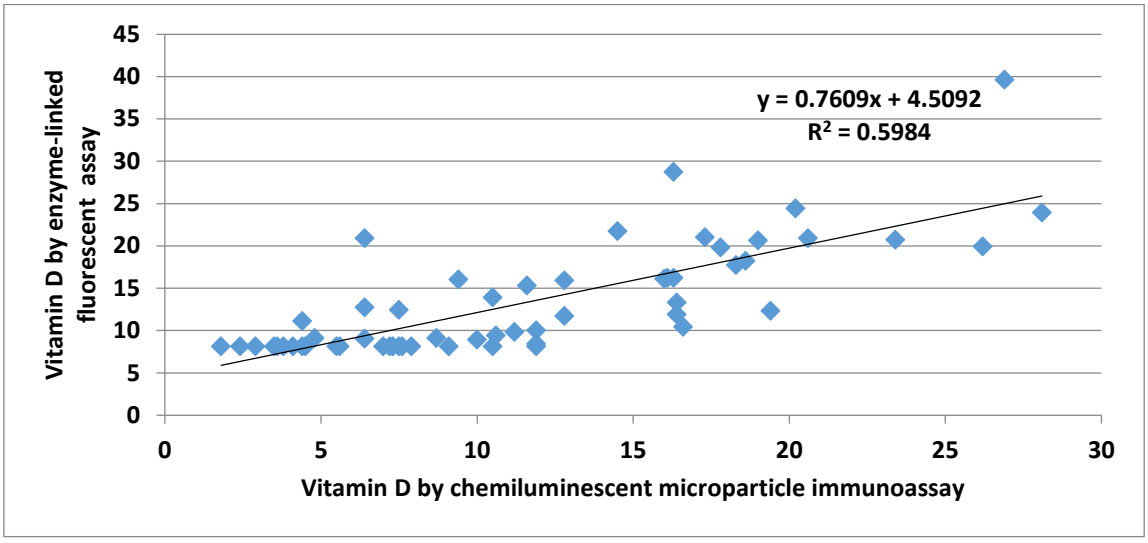

Fig 1. Correlation between the two methods of vitamin D assay; chemiluminescent microparticle immunoassay and enzyme-linked fluorescent assay in a sample of normal subjects (2-tailed Pearson correlation, P < 0.001).

3. Categorization of vitamin D serum level into deficient, insufficient and sufficient ranges.

In both methods of assay, the majority of participants (more than 80%) had serum vitamin D levels within the deficient range, when the cut-off point for deficiency was below 20 ng/ml. This percentage was reduced to around 46% when the cut-off point for deficiency was taken below 10 ng/ml (Table-3).

Table 3. Categorization of vitamin D serum levels into deficient, insufficient and sufficient, when the cut-off points for deficiency was taken as < 20 ng/ml and < 10 ng/ml.

25 (OH)D	METHOD OF ASSAY	CUT-OFF POINT < 20 NG/ML	CUT-OFF POINT < 10 NG/ML
Deficient < 20 ng/ml	CMIA	51 (89.5%)	26 (45.6%)
	ELFA	47 (82.5%)	27 (47.4%)
Insufficient 20-30 ng/ml	CMIA	6 (10.5%)	25 (43.9%)
	ELFA	9 (15.8%)	20 (35.1%)
Sufficient > 30 ng/ml	CMIA	0	6 (10.5%)
	ELFA	1 (1.8%)	10 (17.5%)

4. Correlation between vitamin D serum level with each of serum calcium, phosphorus, alkaline phosphotase and parathyroid hormone.

The correlation between vitamin D serum concentration (measured by both CMIA and ELFA methods) and serum levels of calcium, phosphorus and alkaline phosphatase was not statistically significant. Parathyroid hormone, on the other hand, showed a significant negative correlation with vitamin D serum concentration in both methods (Figure 2, Figure 3).

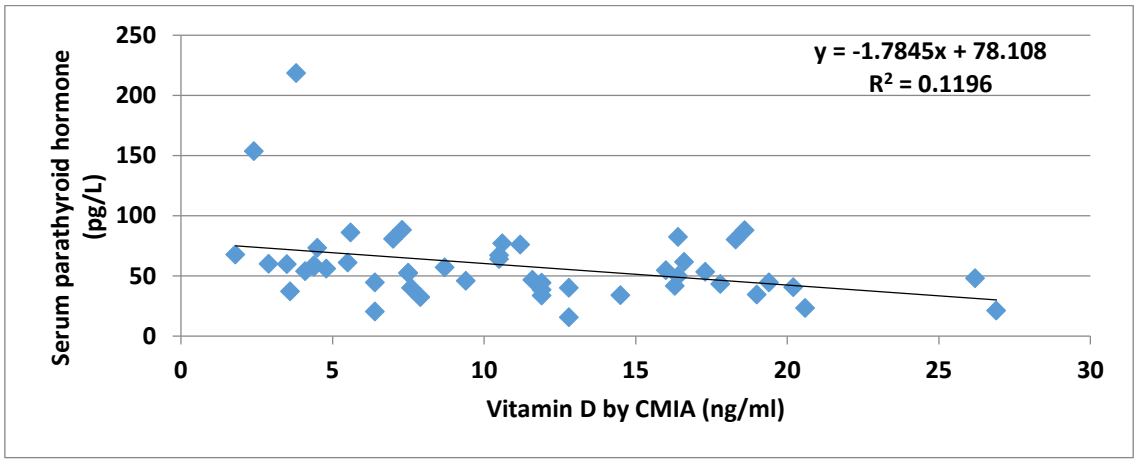

Fig 2. Correlation between vitamin D serum concentration measured by chemiluminescent immunoassay, and serum level of parathyroid hormone in a sample of normal subjects (a significant negative correlation, *P= 0.015*).

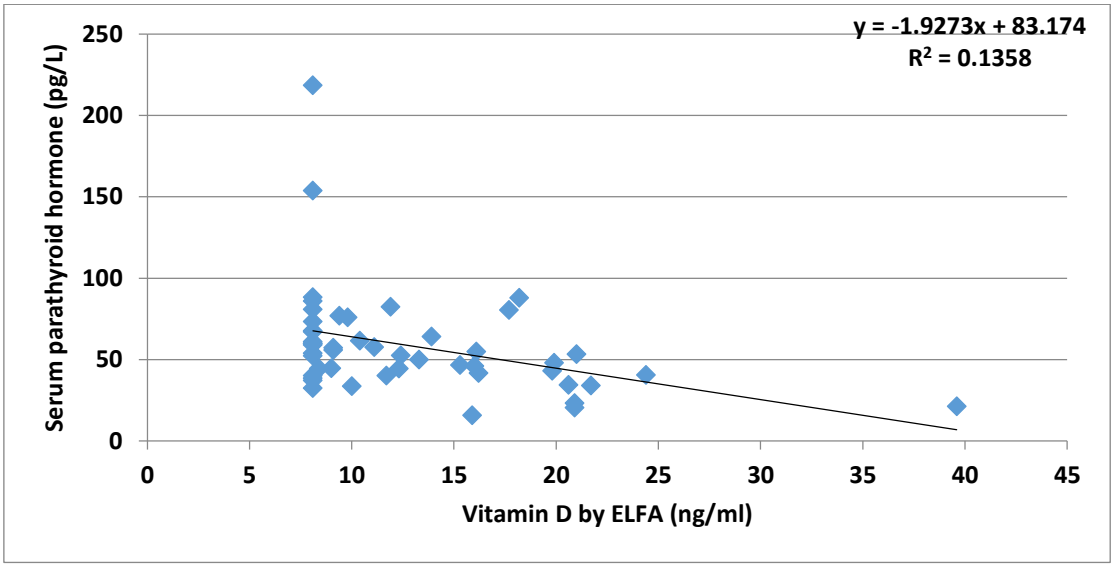

Fig 3. Correlation between vitamin D serum level measured by ELFA and parathyroid hormone in a sample of normal subjects (with a significant negative correlation, P= 0.009).

5. Relationship between different variables and the mean level of serum vitamin D level in a sample of normal subjects.

Variables that showed statistically significant differences in the means of vitamin D serum levels in at least one method of vitamin D assay include: age (significantly lower level in those below 18 years of age when it was measured by ELFA method), gender (significantly higher level in males than females), occupation (significantly higher levels in outdoor

occupations than indoor ones), sun exposure (significantly higher level of vitamin D in > 60 minutes/day than < 20 minutes/day sun exposure), and skin color (significantly higher vitamin D level in dark brown complexion than light brown complexion). After excluding children and females, no significant difference was found between adult smokers and non smokers when vitamin D was measured by both methods (Table-4).

Table 4. Variables that contributed to the level of serum vitamin D level.

Variable	Subgroups	CMIA	ELFA
Age	< 18 years (n=9)	9.02 ± 5.32	10.42 ± 3.92
	≥ 18 years (n=48)	12.05 ± 6.79	13.86 ± 6.80
	Significance	P = 0.158	*P = 0.049*
Gender	Males (n=24)	16.49 ± 6.22	17.77 ± 7.20
	Females (n=33)	8 ± 4.22	10.07 ± 3.39
	Significance	*P = 0.000*	*P = 0.000*
Sun exposure	Sun exposure <20 minutes/day(n=14)	*8.35 ± 4.08*	*10.99 ± 3.78*
	Sun exposure >60 minutes/day(n=22)	*14.55 ± 6.92*	*15.83 ± 6.35*
	Significance	*0.016*	*0.086*
Skin color	Light brown (n=34)	*10.38 ± 5.83*	*12.27 ± 4.87*
	Dark brown (n=7)	*19.61 ± 4.70*	*19.75 ± 6.39*
	Significance	*0.002*	*0.015*
Occupation	Indoor (n=50)	*10.66 ± 6.27*	*12.4 ± 6.27*
	Outdoor (n=7)	*18.1 ± 5.7*	*19.8 ± 4.35*
	Significance	*0.012*	*0.003*
Smoking	Adults male smokers (n=8)	17.82 ± 5.94	*16.66 ± 5.35*
	Adults male non-smokers (n=9)	16.16 ± 6.51	*16.82 ± 5.06*
	Significance	*0.591*	*0.951*

DISCUSSION

Vitamin D deficiency was found highly prevalent, where more than 80% had serum levels of vitamin D below 20 ng/ml, in both methods of assay. When the other cut-off point (< 10 ng/ml) was used, around 46% were found deficient. The prevalence of vitamin D deficiency varied not only between different countries, but even among studies from the same country. It may be as low as 3%[16] and 11.3%[15] or as high as 87.8%[13] and 98%.[21] Using the cut-off point of < 50 nmol/L (i.e. < 20 ng/ml) in another cross sectional study that included 103 females and 95 males, the prevalence of vitamin deficiency was very high in females (around 99%) and males (92.6%).[25] These results are similar to the results of the present study where vitamin D deficiency was found in 100% of female subjects and 75% of males, when vitamin D was measured by the chemiluninescent method. Different cut-off points, different methods of assay, seasonal and circadian variation and different characteristics of the study population may be behind such variations. Various risk groups might be expected to have different cut-off points. For example, breast-fed infants versus formula-fed,

children versus adolescents, adults, or elderly, dark versus light skin, adequate versus inadequate sun exposure, obese versus non-obese, and males versus females. However, nothing, in the reviewed literature, is clear about the use of specific cut-off points for different risk groups. The same cut-off points were used no matter what the characteristics of the participants were. The present study showed that the mean of vitamin D serum level was significantly lower among those aged less than 18 years in comparison to those 18 years and above when it was measured by ELFA method. The low level of vitamin D among children and adolescent had also been reported previously.[26] The latter study showed that all study participants among children had vitamin D deficiency. This was attributed to a decline in physical activity with less sun exposure. In addition, the peak of bone mass during puberty requires more vitamin D, resulting in a decline in its level.[26] It was found that 28% of young men had insufficient vitamin D, the percentage increased to 37% among those aged > 50 years.[2] Reduced skin ability to produce vitamin D with increasing age, was a possible explanation for low level of vitamin D among elderly people.[17] The present study revealed that mean level of vitamin D was significantly lower among females when compared with males. Junaid et al[27] considered female gender as a risk factor for vitamin D deficiency. This might be related to less sun exposure due to decreased outdoor activities, and to wearing veils.[17] Nearly all female participants in our study were wearing veils. Estrogen had been found to stimulate 1α-hydroxylase enzyme in the kidney which is responsible for the conversion of 25(OH)D to the active $1,25(OH)_2D$. This results in an increase in $1,25(OH)_2D$ level and a decrease in 25(OH)D level.[28] The present study showed that participants with indoor occupations had significantly lower levels than those of outdoor ones. Sowah et al[29] also found that 78% of indoor workers were vitamin D deficient, versus 48% among outdoor workers. One obvious explanation is lack of enough sun exposure. Smoking as enzyme inducer, is expected to stimulate vitamin D metabolism and contribute to its deficiency. Ren et al[30] found that the level of vitamin D in smokers was significantly lower than non-smokers. Similarly, Jiang et al[31] when compared current and never smokers that vitamin D level was lower among current smokers, with an inverse relationship between level of vitamin D and number of cigarettes per day and smoking duration. Shinkov et al[32] also reached to a similar conclusion in male smokers. In contrast to the above-cited findings, in the present study, it was found that serum level of vitamin D was higher among adult male smokers when compared with non-smokers. This unexpected result, although statistically not significant, might be related to the outdoor lifestyle of smokers with sufficient sun exposure. A Similar conclusion was reached by Grimnes et al[33], where current smokers had higher levels of vitamin D in comparison to never smokers. In addition to that, a number of paradoxical results had been encountered in the present study. Vitamin D is a fat-soluble vitamin. However, it was found that as the BMI increased, the prevalence of vitamin D deficiency decreased. People with darker skin such as African Americans or Hispanics were reported to have much lower vitamin D levels than those with lighter skin.[34] The reverse was true in the present study, where those with darker skin had significantly higher vitamin D serum levels. This dark skin, was not the black race, but might reflect sufficient sun exposure and more vitamin D synthesis. Finally, it can be concluded that vitamin D deficiency is common (> 80%) among apparently normal subjects in Basrah; the mean level was in the deficient range if the cut-off point was taken as 20 ng/ml. This prevalence is reduced to around 46% when the cut-off point was taken as 10 ng/ml, which might reflect the true situation more

appropriately. The two methods used to measure vitamin D level were well correlated but the enzyme-linked fluorescent assay resulted in higher mean level than chemiluminescent assay. A search for appropriate cut-off point that accurately describes vitamin D deficiency is recommended. Also, to compare the widely used immunoassay methods with more specific methods like HPLC and LC-MS/MS and to study how much of skin area should be exposed to sunlight to keep serum vitamin D within normal range.

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