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Original article

Vitamin D and LL-37 in children with pneumonia

Background: *Vitamin D is involved in the regulation of about1000 human genes. Recent studies suggest that vitamin D may have other actions outside of its classic functions related to bone and calcium homeostasis. The only human cathelicidin, LL 37, enhances microbial killing against a broad range of respiratory pathogens and has a defined vitamin D- dependent mechanism.* ***Objective****: evaluate the association between vitamin D status and plasma LL-37 levels in children with pneumonia.* ***Study design****: Forty consecutive children admitted to the chest unit of the Pediatric Department of Zagazig University Hospital with proven diagnosis of pneumonia were included in the study. They were 22 males and 18 females, of ages ranging from 2 to 5 years. In addition, 40 age and sex matched apparently healthy children served as a control group. A verbal consent was obtained from parent(s) of each child before inclusion to the study. All children were subjected to history taking, clinical examination, routine investigations (CBC, CRP and ESR) and chest X-ray done for patients only, as well as determination of serum 25-OH vitamin D and plasma LL-37.* ***Results:*** *The study revealed a highly significant increase of WBCs, ESR and CRP and a highly significant decrease in hemoglobin of patients. Absence of history of sun exposure, increased WBCs counts and low levels of vitamin D and LL-37 were considered as risk factors for pneumonia while site of residence, hemoglobin level and platelets count were not. Both vitamin D and LL-37 were significantly lower in patients than controls (P<0.001). There were significant positive correlations between vitamin D and LL-37 in studied groups.* ***Conclusion:*** *Appropriate concentrations of vitamin D facilitate the ability of immune system to defend against respiratory tract infections through enhancing LL-37 production.*

Key word: vitamin D- LL 37- pneumonia

Ehab AM. Albanna, Yasser F. Ali, Reayid AM. Elkashnia*

Departments of Pediatrics and Biochemistry, Faculty of Medicine, Zagazig University.*

Correspondence:
Ehab Abdel Moneim Albanna. Pediatric Department, Zagazig University, Zagazig, Sharkia governorate, Egypt.
E-mail: *ehab-banna @yahoo.com*

INTRODUCTION

Until recently, many health care professionals believed that the major health problems resulting from vitamin D insufficiency were limited to bone health, including rickets, osteomalacia, and osteoporosis. Recent studies suggest that vitamin D may have other actions outside of its classic functions related to bone and calcium homeostasis[1].

Vitamin D is involved in the regulation of 1000 human genes[2]. Because few foods contain vitamin D, sunlight exposure is the primary determinant of vitamin D status in humans[3].Vitamin D synthesis is initiated in the skin by UVB radiation from the sun activating its precursor 7-dehydrocholesterol, which then circulates in the blood to the liver, where it is converted into its main metabolite 25-hydroxyvitamin D '25(OH)D' which has blood level about 1000 times higher than the active metabolite, 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$)[4].

It was thought that the conversion to 1, 25-$(OH)_2D$ occurred only in the kidneys, but increasing evidence indicate that the cells of most organs have vitamin D receptor and, along with this, the capacity to synthesize 1, $25(OH)_2D$ locally. This autocrine and paracrine synthesis of 1, $25(OH)_2$ D is dependent on serum 25(OH)D levels, the primary circulating form of vitamin D1.

Until recently, serum 25(OH)D levels of at least 25-50 nmol/L appeared to be adequate, based on the absence of rickets and improved skeletal outcomes, but increasing evidence suggests that the levels of at least 75 nmol/L are required for good health[5]. Emerging evidence indicate that vitamin D-mediated innate immunity, particularly through enhanced expression of the human cathelicidin antimicrobial peptide (hCAP-18) is important in host defenses against respiratory tract pathogens[6,7].

Vitamin D insufficiency is widespread and is associated with increased incidence of respiratory tract infections in preliminary studies[8,9]. Cells of the

innate and adaptive immune system including macrophages, lymphocytes and dendritic cells express the vitamin D receptor (VDR) and respond to stimulation by1,25$(OH)_2D$[10].

Cathelicidin, known as LL-37; which is cleaved from its precursor hCAP18 is an endogenous antimicrobial peptide active against a broad spectrum of infectious agents including gram negative and positive bacteria, fungi, mycobacteria and viruses by acting as chemoattractant for neutrophils and monocytes, and has a defined vitamin D-dependent mechanism[10,11].

LL-37 is highly expressed at barrier sites including respiratory and colonic epithelium, saliva, and skin and thus provides an important first line defense mechanism for the innate immune system to respond to infectious insults[7]. The addition of 25(OH)D to the media up-regulated production of LL-37 suggesting that vitamin D has an important role in the production of anti-microbial peptides important for innate immunity[7].

Acute lower respiratory infection, primarily pneumonia, is a common cause of morbidity and mortality in children younger than 5 years of age, particularly in developing countries[12].

Clinical vitamin D deficiency (rickets) was associated with 13-fold increased risk of pneumonia in Ethiopian children less than 5 y of age[13].

However, we are unaware of studies that have investigated the role of subclinical vitamin D deficiency in respiratory tract infections among infants and young children in developing countries. As subclinical vitamin D deficiency is common even in countries with plentiful sunshine, this work aimed to evaluate the association between vitamin D status and plasma LL-37 levels in children with pneumonia.

METHODS

This case control study included 40 children (22 males and 18 females) with proven diagnosis of pneumonia (7 had viral pneumonia with lymphocytosis and negative CRP and 33 had bacterial pneumonia with neutrophilia and positive CRP) of ages ranging from 2 to 5 years (X±SD: 3.35± 0.9 years) who were admitted to chest unit of Pediatric Department, Zagazig University Hospital during the year 2009 through 2010.

Exclusion criteria

- Children under the age of 2 years.
- Recurrent pneumonia, defined as two episodes or more in one year or more than three episodes of pneumonia in a child at any time, with radiographic clearing between episodes [14].
- Infants with clinically diagnosed vitamin D deficiency rickets.
- Children who needed ventilator or died from pneumonia.

All patients were given IV ampicillin-sulbactam (in a dose of 150 mg/kg) divided every 12 hours and IV cefotaxime (in a dose of 100mg/kg) in 2 divided doses. Typically patients with uncomplicated community-acquired bacterial pneumonia respond to therapy with improvement in clinical symptoms (fever, cough, tachypnea, chest pain) within 48-96 hours of initiation of antibiotics. Radiographic evidence of improvement substantially lags behind clinical improvement. A number of factors must be considered when a patient does not improve on appropriate antibiotic therapy (slowly resolving pneumonia)[15].

Forty healthy children (20 males and 20 females) of mean ages 3.4± 1.1 years were studied as control group.

Ethical approval was obtained from the local research ethics committee and parents of all children gave an informed written consent prior to the study.

All children were subjected to the following:

1- History taking including socio-economic level, residence, sun exposure and history of upper respiratory tract infection
2- Clinical examination including body temperature, respiratory rate, cyanosis and local chest examination.
3- Routine laboratory investigations including complete blood count (CBC), C-reactive protein (CRP)[16] (positive above 6 mg/L) and erythrocyte sedimentation rate (ESR).
4- Chest X ray (for patients only).
5- Measurement of serum 25OHD: Two ml of venous blood were obtained centrifuged and serum was separated and stored at -20ºC until assayed. Serum level of 25OHD was measured after extraction using the immunodiagnostic enzyme immuno-assay (EIA) developed by Immuno-diagnostic, Bensheim and Biomedica, Wien Australia[17]. Catalog number 02082005 25 OH vit D6.DOC. The cut off level of vitamin D was 35 nmol/l.
6- Measurement of human plasma LL-37: Tow ml venous blood sample was collected on EDTA, centrifuged and plasma was separated. The plasma stored at -70ºC in polypropylene tubes until assay. The human LL-37 was measured using solid-phase enzyme linked immunosorbent assay (ELISA) based on the sandwich principle using a commercial human LL-37 ELISA kit, HK321 Hycult Biotech, Fronststraat 2a, 5405PB

Uden, the Netherlands[18]. The cut off level of LL 37 was 20 ng/ml.

Statistical analysis

Data were presented as mean ± standard deviation (X±SD) or percentage (%). The means of two groups were compared using student "t" test. Linear correlation and regression were used to test the correlation between the measured parameters. Odds ratio was used to quantify the risk. Cut of values were calculated from the ROC curve as mean ± 2SD of control. Data were tabulated and statistically analyzed with the statistical package for Social Sciences (SPPS) version 14 software. P-values less than 0.05 were considered significant[19].

RESULTS

Analysis of demographic characteristics of the studied groups revealed that there were nonsignificant differences between patients and controls as regard age, sex, socioeconomic state and residence (Table 1).

Table 2 shows the laboratory data of 40 children with pneumonia versus 40 control children. WBCs, ESR and CRP were significantly higher in patients than controls while hemoglobin levels were significantly lower.

Absence of history of sun exposure, high WBCs counts and low levels of both 25OHD and LL-37 were considered risk factors for pneumonia while site of residence, hemoglobin level and platelet counts did not (Table 3).

Table 4 shows that both vitamin D and LL-37 were significantly lower in children with pneumonia than in control group.

This study showed nonsignificant differences between children with resolved and those with slowly resolved pneumonia regarding vitamin D and LL-37 (Table 5).

Our study showed a highly significant positive correlation between vitamin D and LL-37 in patients, control and in children with resolved pneumonia and a significant positive correlation in children with slowly resolved pneumonia (Table 6).

Table 1: Demographic characteristics of the studied groups

Variables	Patients (*n*=40)		Control (*n*=40)		*t*	χ^2	*P*
Age (years) X±SD	3.35±0.9		3.4±1.1		0.21	-	0.82
Sex (*n*, %)							
Male	22	55.5	20	50.0	-	0.2	0.62
Female	18	45.0	20	50.0			
Socioeconomic level (*n*, %)							
Low	28	70.0	20	50.0	-	3.33	0.067
Middle	12	30.0	20	50.0			
Residence (*n*, %)							
Urban	15	37.5	14	35.0	-	0.05	0.81
Rural	25	62.5	26	65.0			

P > 0.05: nonsignificant

Table 2: Laboratory characteristics of the studied groups

Laboratory investigations	Patients (*n*=40) X±SD (Range)	Control (*n*=40) X±SD (Range)	*t*	*P*
WBCs ($\times 10^3/mm^3$)	15.76±3.4 (10.2 – 31.9)	7.85±1.6 (5.7- 10.3)	13.2	<0.001
Hemoglobin (g/dL)	11.2±0.7 (10 -12.7)	12.4±1.3 (10 -15)	5.23	<0.001
Platelets count ($\times 10^9$/L)	221.9±17.9 (191- 260)	217.4±22.3 (183 – 256)	0.98	0.67
ESR (mm/hr)	33.1±6.4 (20 – 45)	11.6±4.1 (6.0 – 19)	17.86	<0.001
CRP (mg/L)				
Positive	33 (82.5)	0 (0.0)	χ^2 =56.17	<0.001
Negative	7 (17.5)	40 (100)		

WBCs: white blood cells, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, *p*< 0.001: highly significant, *p* >0.05 : nonsignificant

Table 3: Odds ratio of the demographic and laboratory data between studied groups

	Patients (*n*=40)	Control (*n*=40)	OR (95% CI)
Residence			
Rural	15 (37.5)	14 (35)	1.11
Urban	25 (62.5)	26 (65)	(0.41-3.06) *
Sun exposure			
Positive	2 (5)	10 (25)	0.16
Negative	38 (95)	30 (75)	(0.02-0.86)**
WBCs count			
Normal	15 (37.5)	34 (85)	9.44
Abnormal	25 (62.5)	6 (15)	(2.89-32.46) **
Hemoglobin level			
Normal	32 (80)	38 (95)	4.75
Abnormal	8 (20)	2 (5)	(0.84-35.08) *
Platelets count			
Normal	40 (100)	40 (100)	-
Abnormal	0 (0)	0 (0)	
Vitamin D (nmol/l)			
<35	31 (77.5)	8 (20)	13.78
>35	9 (22.5)	32 (80)	(4.21-47.46) **
LL-37(ng/ml)			
<20	31 (77.5)	10 (25)	10.33
>20	9 (22.5)	30 (75)	(3.31-33.62) **

OR: odds ratio, CI: confidence interval, **: Highly significant, *: Nonsignificant

Table 4: Vitamin D and LL-37 among the studied groups

	Patients (*n*=40)	Control (*n*=40)	*t*	*P*
Vitamin D				
X±SD	37.6±21.1	87.25±18.4	11.19	<0.001
Range	20 - 95	40 - 120		
LL-37				
X±SD	14.7±6.2	22.0±2.5	6.97	<0.001
Range	5 - 25	15 - 25		

p< 0.001: highly significant

Table 5: Vitamin D and LL-37 according to resolution of pneumonia

	Resolved (*n*=32) X±SD (Range)	Slowly resolved (*n*=8) X±SD (Range)	*t*	*P*
Vitamin D	34.87±16.6 (20-80)	48.6±33.1 (20-95)	1.68	0.09
LL-37	15.1±6.2 (5-25)	13±6.2 (5-20)	0.85	0.59

p >0.05: nonsignificant

Table 6: Correlation between vitamin D and LL-37 in studied groups

	r	*P*
Control	0.76	<0.001
Patients	0.6	<0.001
Resolved pneumonia	0.72	<0.001
Slowly resolved pneumonia	0.66	<0.05

p< 0.001: highly significant, *p* <0.05 : significant

DISCUSSION

In our series, patients had significantly lower 25OHD levels than controls which can be explained by history of inadequate sun exposure in our patients.

In this study, there were nonsignificant differences between patients and controls regarding age, socioeconomic state and site of residence to explain the low levels of 25OHD in the patient group and this is in agreement with an Indian study which revealed non significant differences between

cases of severe acute lower respiratory infections and controls in mean ages or distribution[20]. Similar results were also reported by other studies[21].

In our study, patients had significantly lower LL-37 levels than controls. The decrease in LL-37 is the result of the decrease in 25OHD. LL37 is produced on epithelial surfaces and within circulating leukocytes that are capable of killing viruses, bacteria, and fungi[22]. Vitamin D (produced in response to sunlight) has recently been shown to have an important role in the innate immune system. It stimulates the secretion of natural antibiotics known as antimicrobial peptides. These peptides include β-defensins and cathelicidins (LL-37)[23]. It has been found that vitamin D has additional defensive roles such as regulating the inflammatory response and chemo-attracting cells of the adaptive immune system to wound or infection sites, binding and neutralizing lipopoly-saccharides, and promoting re-epthelialization[11,24]. Ensuring optimal vitamin D levels -through appropriate sunlight exposure, diet, if necessary, supplementation- is likely to help ensure optimum protection from infectious diseases. This has particular relevance in the winter as vitamin D levels tend to bottom out. In fact, lower level of vitamin D may well be a factor in why it is that infectious diseases such as cold, flu and pneumonia tend to be more common in the winter[25,26] .This gives vitamin D the potential to combat a range of infections. Bartley[27] and Ginde et al.[28] reported that vitamin D is involved in the production of defensins and cathelicidin which provide a natural defense against potential microbiological pathogens and concluded that vitamin D supplementation increases cathelicidin production. Also, Jeng et al.[26] declared that plasma LL-37 levels were significantly lower in critically ill patients with and without sepsis compared to the healthy controls.

In support of the role of 25OHD in the production of LL-37, the present study revealed a significant positive correlation between vitamin D and LL-37 in control children (r=0.76) and patients with resolved pneumonia (r=0.72), as well as in patients with slowly resolved pneumonia (r=0.66).

A similar positive correlation was also found by Jeng et al.[26].

The finding of significantly higher WBCs, ESR and CRP and significantly lower hemoglobin levels in patients compared to controls is a well known response in patients with pneumonia as has been reported by other investigators[15].

In our study, positive previous history of sun exposure, normal WBC count, normal 25OH D level >35 nmol/L and normal LL-37 level >25 ng/ml carry a significantly lower risk for development of pneumonia in children while site of residence, hemoglobin level and platelet count did not have any risk. Similar results were reported by Wayse et al.[20] who declared that factors significantly associated with decreased risk of severe acute lower respiratory infection in univariate analysis were: infant not covered in swaddling clothes when exposed to sunlight before crawling and serum 25OHD3 >22.5 nmol/l . Also, a Finnish cohort study found that young male soldiers with serum 25OHD levels less than 40 nmol/l at baseline had a 63% increased risk of absence from duty due to respiratory infections over the following 6 months than soldiers with levels ≥ 40 nmol/L[8]. Additionally a Turkish case control study found that serum 25OHD levels were lower in neonatal cases of acute lower respiratory infection (22.8 nmol/l) than in age-matched controls (40.8 nmol/l)[28]. In contrast to our result, a Canadian case control study of children 1 to 25 months old found no difference in mean serum 25OHD levels between patients with acute lower respiratory tract infection (77.0 nmol/l) and hospital controls (77.2 nmol/l), the average vitamin D status of these individuals was greater than 75 nmol/l, which can be explained by that all the studied infants consumed vitamin D through fortified infant formula or supplements[25].

In this study, we did not find any significant difference in the mean serum vitamin D and LL-37 levels between patients with resolved and those with slowly resolved pneumonia. Slow resolution can be explained by a number of other factors such as bacterial resistance, non bacterial etiologies such as viruses and aspiration of foreign body or food, mucous plugs and pre-existing diseases such as immunodeficiencies, ciliary dyskinesia, cystic fibrosis, pulmonary sequestration, or cystic adenomatoid malformation, that must be considered when a patient does not improve on appropriate antibiotic therapy[15] .This result can be explained by that LL-37 plays a role in the defense against infection but once infection has taken place, other factors determine its course and whether it will resolve or not.

In conclusion, inappropriate concentrations of vitamin D decrease the ability of the immune system to defend against respiratory infections through lowering LL-37. So, we recommend appropriate vitamin D supplementation and sun exposure to decrease the risk of respiratory tract infections and performing further studies to define the exact relation of vitamin D status to other infections.

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