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Vitamin B12 and D Deficiencies Up-regulate the production of TNF-α from TLR2 in malnourished Egyptian children

Salwa Refat El-Zayat[1], Hiba Sibaii[1], Hanan Saleh[2], Shebl Shaalan[2], Salwa M. Saleh[3], Nevein N. Fadl[1], Mahmoud M. ElHefnawi[1] and Hanan S. kiwan[1]**

[1] Medical Physiology Department, National Research Centre, 12311, Giza, Cairo, **Egypt**,
[2] Zoology Department, Faculty of Science, Cairo University, 12316, Giza, Cairo, **Egypt**
[3] Outpatient Clinic, National Nutrition Institute, Giza, Cairo, **Egypt**

***Correspondence:** hebead@sci.cu.edu.eg, hanan_ebead@yahoo.com **Accepted:** 25 Dec. 2017 **Published online:** 28 Feb. 2018

Background: Although vitamins B_{12} and D are well known immune modulators, they have less medical interest than they worth. Aim: we aimed to evaluate the effect of vitamin B12 and D deficiencies on the function of toll like receptor 2 (TLR2) in malnourished children. Subjects and Methods: 87 children were divided according to their plasma concentrations of vitamin B12 and D into four groups: B12 (group 1; sufficient & deficient subgroups), D (group 2; sufficient & deficient subgroups), B12 & D sufficient (group 3) and B12 & D deficient (group 4). Heparinized blood was cultured by PAM3CSk4 for 24 hours to stimulate TLR2 then the released concentration of tumor necrosis factor alpha (TNF-α) was determined by ELISA array. Results: There was a significant difference in vitamin B12 levels in group 1 and vitamin D levels in group 2 at p< 0.001. There was a significant increase in TNF-α levels in group 1 and group 2 in vitamin B12 and vitamin D deficient subgroups when compared to their corresponding sufficient subgroups at $p<0.05$ and $p<0.006$ respectively. In addition we observed a significant increase in TNF-α level in group 4 when compared to group 3 at $p<0.001$. Our results revealed a negative correlation between TNF-α and both of vitamin B12 and D at (r= - 0.262, $p< 0.05$) and (r= -0.385, $p< 0.004$) respectively. Conclusion: Deficiencies in vitamins B12 and D may influence the function of TLR2 by increasing its cytokine output of TNF-α in Egyptian malnourished child.

Keywords: Toll-like receptor 2; vitamin B12; vitamin D; malnutrition; TNF-α

INTRODUCTION

Vitamins B12 and D have been known as essential immunomodulators due to their anti-inflammatory functions. Vitamin D receptor (VDR) was found in most cells of the immune system such as macrophages, monocytes, activated B cells and all lines of T cells (Cantorna et al., 2015; Goldsmith, 2015). Insufficient vitamin D might cause pro-inflammatory stress (Barker et al., 2013). On the other hand, deficiency in vitamin D is correlated with the increased risk for chronic inflammation, infections and autoimmune diseases (Slusher et al., 2015; Sommer and Fabri, 2015). It is well known that vitamin B12 improves the overall function of the immune system, prevents excessive expression and synthesis of inflammatory cytokines and its deficiency influences the susceptibility of infectious diseases (Bhaskaram, 2002; Badawi et al., 2013). The production of pro-inflammatory cytokines is rigorously controlled and adjusted by variety of immune recognition receptor families, the best described of which is the toll-like receptors family (TLRs). Upon stimulation, TLRs activate

downstream cascades that leading to the release of various cytokines and immune modulators (Takeda and Akira, 2005; Kharaji and Haghparast, 2010). Several studies have given many evidences on the contribution of TLRs signaling dysregulation to the development and progression of numerous diseases such as infectious, chronic inflammatory and autoimmune diseases. TNF-α is a pro-inflammatory cytokine known as the key initiator of immune mediated inflammation (Komurcu et al., 2016). TLR2 is extensively distinguished member that induces the production of TNF-α (Gambhir et al., 2012; Schnetzke et al., 2015) and its dysregulation is linked with many health complications (So and Ouchi, 2010; Lu et al., 2014). TNF-α is released after exposure to various inflammatory stimuli, mediates the catabolic reactions and induces illness (Bresnahan and Tanumihardjo, 2014). Therefore, this study aimed to assess the effect of vitamins B12 and D deficiencies on the function of TLR2 in Egyptian malnourished children.

MATERIALS AND METHODS

This study was performed in the National Research Centre (NRC) and the National Nutrition Institute (NNI), Egypt, during the period from June to August 2016. The protocol and procedures used in this study were carried out in accordance with the international guidelines of the world medical association (Declaration of Helsinki) and approved by the "Medical Research Ethics Committee" of "NRC" (approval ID:15072) before the start of the study. Children were recruited from the outpatient clinic of the NNI; a written consent form was taken from the legal guardian of each child enrolled in the study. They underwent complete clinical examination in order to exclude volunteers with organic and genetic disorders that might interfere with normal growth. Selected children were subjected to some anthropometric measurements, they were weighed to the nearest 0.1 kg using a standardized platform scale and height was measured to the nearest 0.5 cm using a Raven Minimeter.

According to the international reference ranges of plasma concentrations of vitamins B12 and D, Habte et al.,(2015) defined vitamin B12 reference ranges as sufficient (> 200 pg/ml), moderately deficient (150-200 pg/ml) and severely deficient (< 150 pg/ml); Holick (2008) defined also the reference ranges of vitamin D as sufficient (≥ 30 ng/ml), insufficient (21-29 ng/ml) and deficient (≤ 20 ng/ml), but in our study, all children recorded plasma concentrations of vitamin B12 < 200 pg/ml. Therefore, we considered the concentrations ranged from 150 to 200 pg/ml were taken as sufficient subjects and those recorded concentrations < 150 pg/ml was taken as deficient ones. Regarding vitamin D they recorded levels matched with the above mentioned international range by Holick (2008).

Accordingly, our subjects were divided into four groups: vitamin B12 (group 1 with two subgroups sufficient and deficient; n= 11 each), vitamin D (group 2 with two subgroups sufficient and deficient; n= 17 each), vitamin B12 and D sufficient (group 3; n=15) and vitamin B12 and D deficient (group 4; n=16).

1- Sample collection:

Five millimeters of venous blood were drawn from each child and divided into two vacutainer tubes, 2 ml in heparinized vacutainer tube for *In vitro* stimulation of whole blood with TLR2 agonist (PAM3CSK4) and 3 ml in (EDTA) tube for biochemical analysis.

2- Sample preparation:

Plasma were separated by centrifugation of blood at 3000 rpm for 10 minutes and stored at -80°C for estimation of vitamin B12 and D concentrations. TLR2 was stimulated as previously described according to Blankley et al., (2014). In each well of 24 wells plates 0.5 ml of heparinized whole blood was diluted with 0.5 ml RPMI-160 supplemented with (1% penicillin/streptomycin, 1% sod. pyruvate and 1% L-glutamin) and stimulated by 300 ng/ml of PAM3CSK4 (Invivogen, Europe) for 24 hours at 37°C and 5% CO_2. After incubation, supernatants were separated in clean sterile eppendorf tubes by centrifugation at 1008 rpm and 4°C for 15 minutes, and then stored at -80°C for determination of TNF-α.

3- Biochemical analysis:

Vitamin B12 was determined by measuring plasma cobalamin using human vitamin B12 ELISA kit, according to the manufacturer instructions (CUSABIO, China,), Plasma 25-hydroxy vitamin D was measured using ELISA kit, according to the manufacturer instructions (DLD Gesellschaftfür Diagnostika und medizinischeGerätembH, Germany, and TNF-α was measured in supernatants using ELISA kit, according to the manufacturer instructions (INOVA, China).

Statistical analysis:

Data were analyzed using SPSS version 16 computer program. One way ANOVA test was used to compare between groups. Person's correlation test was used to estimate the linear correlation between variables. Data were expressed as the mean ± SD or mean ± SE post hoc LSD. Statistical significance were set at p <0.05.

RESULTS

87 children aged from 5 to12 years were enrolled in this study. According to gender, children were divided to 56 males representing 61.0% and 31 females representing 39.0% of the study. The distribution of the children according to sex and their ages and body mass index (BMI) were showed in table (1) and table (2), respectively. Plasma concentrations of vitamins B12 and D in addition to TNF-α in the different groups were showed in table (3) and table (4).

Table (1): Distribution of the children according to sex

Gender	Group 2 (n=34) (39.0%)	Group 1 (n=22) (25.28%)	Group 3 (n=15) (17.24%)	Group 4 (n=16) (18.39%)	Total (n=87)
Males	26 (76.5%)	14 (63.6%)	9 (60.0%)	7 (43.8%)	56 (61.0%)
Females	8 (23.5%)	8 (36.4%)	6 (40.0%)	9 (56.3%)	31 (39.0%)

Values are expressed as mean±SE , NS: non-significant

The values was significant different p<0.05

Table (2): Age and Body Mass Index (BMI) of the children

Parameter	Group 1 (n=22)	Group 2 (n=34)	Group 3 (n=15)	Group 4 (n= 16)
Age (mean±SD)	7.6±3.1	7.2±2.9	9.0±1.7	7.0±2.8
***P* value**		0.7 NS	0.21NS	0.59 NS
BMI (mean±SD)	17.6±7.43	14.77±4.47	21.41±8.62	21.0±7.5
***P* value**		0.69 NS	0.90 NS	0.63 NS

Values are expressed as mean±SE , NS: non-significant

The values was significant different p<0.05

Table (3): Plasma concentrations of vitamin B12 and D in groups 1 and 2 and the concentrations of TNF-α produced upon stimulation with PAM3CSK4

Groups	Group 1(mean±SE) (n=22)		P value	Group2(mean±SE) (n=34)		*P* value
parameters	vit.B12(pg/ml) sufficient (n=11)	vit.B12 (pg/ml) deficient (n=11)		vit.D (ng/ml) sufficient (n=17)	vit.D (ng/ml) deficient (n=17)	
	157.91±2.48	89.47±6.8	p<0.001	41.3±4.3	14.42±0.76	p<0.001
TNF-α (pg/ml)	38.90±2.16	49.5±2.26	p<0.05	41.1±1.68	50.7±2.90	p<0.006

Values are expressed as mean±SE , NS: non-significant

The values was significant different p<0.05

Table (4): TNF-α concentration in groups 3 and 4 produced upon stimulation with PAM3CSK4

Groups	Group 3 (mean±SE) vit.B12&D sufficient (n=15)		Group4 (mean±SE) vit. B12&D deficient (n=16)		*P* value
parameters	vit.B12 (pg/ml) sufficient	vit.D (ng/ml) sufficient	vit.B12 (pg/ml) deficient	vit.D (ng/ml) deficient	
	157.13±2.44	35.61±2.74	83.12±6.68[a]	14.7±0.72[b]	
TNF-α (pg/ ml)	38.28±2.1		51.7±3.31		p<0.001

Values are expressed as mean±SE , NS: non-significant

The values was significant different p<0.05

a: significance of vit.B12 sufficient group in comparison to vit.B12 deficient group

b: significance of vit.D sufficient group in comparison to vit.D deficient group

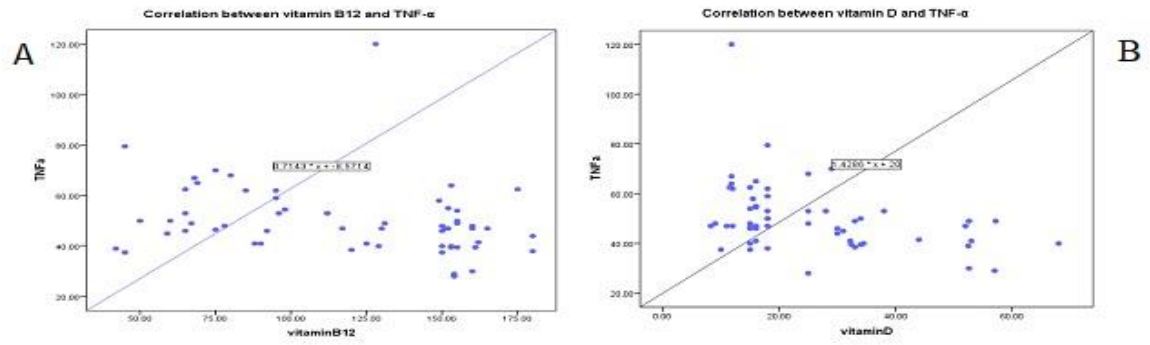

Figure. (1): Correlation between vitamin B12 (A) and TNF-α (B).

As shown in table 2 there was a significant difference in vitamin B12 levels in group 1 and vitamin D levels in group 2 at $p< 0.001$. In group 1 and group 2, regarding TNF-α level, it has shown an increased levels in vitamin B12 and vitamin D deficient subgroups in comparison to their corresponding sufficient subgroups at $p<0.05$ and $p<0.006$ respectively. In addition we observed a significant increase in TNF-α level in group 4 when compared to group 3 at $p<0.001$. Our results revealed negative correlation between TNF-α and both of vitamin B12 and D at (r= -0.262, $p< 0.05$) and (r= -0.385, $p< 0.004$) as shown in figures (1) and (2), respectively

DISCUSSION

Vitamins B12 and D have been known as anti-inflammatory immunomodulators (Raman et al. 2011; Hosseinzadeh et al., 2012). Our study showed that the deficiencies in vitamins B12 and D are associated with the increased production of TNF-α. Vitamin B12 deficient group released significantly higher amount of TNF-α compared to the sufficient group ($p<0.05$), this was in accordance with the results by Scalabrino et al., (2004) who found the same results in the cerebrospinal fluid and Ghatpande et al., (2016) who observed a high amount of TNF-α in the plasma of adolescent girls deficient in vitamin B12.

In our study there was an inverse correlation between vitamin B12 and TNF-α that could be attributed to inhibitory effect of cobalamin on nitric oxide synthase and nitric oxide production that control NF-κB activation (Manzanares and Hardy, 2010). This observation was confirmed by Hosseinzadeh et al., (2012) who proved the anti-inflammatory effects of vitamin B12 in acute and chronic inflammation in mice.

Our study showed that vitamin D deficient children produced significantly higher amount of TNF-α than that produced by the sufficient group ($p< 0.06$). This was in agreement with Ojaimi et al. (2013) who showed the increase in TNF-α production in vitamin D deficient participants and after supplementation with vitamin D reduction of TNF-α was observed in those participants. In addition a recent study released by Hoe et al. (2016) showed a significant reduction in TNF-α production after of the addition of vitamin D to the cultured cells.

The present results showed a negative correlation between TNF-α and vitamin D. This could be attributed to the immunomodulatory role of vitamin D. Vitamin D was reported to suppress the Th1 cells by down regulating the Th1-polarizing cytokine TNF-α (Sundaram and Coleman, 2012; Pettengill et al., 2014). Vitamin D suppresses TNF-α by down regulating the expression of TLR2 on the surface of immune cells (Akcakus, 2006; Do et al., 2008) and suppressing the signaling through it by inhibiting some downstream proteins such as NF-κB (Coussens et al., 2014).

In our study, children deficient in both vitamin B12 and vitamin D released the highest amount of TNF-α ($p<0.001$) whereas those sufficient in both vitamins produced the lowest amount of TNF-α. Thus, our results suggested that vitamins B12 and D synergistically modulate the inflammatory response where the deficiency of one vitamin can be partly compensated by the existence of the other whereas the deficiency of both vitamins markedly increased TNF-α.

Therefore, we concluded that deficiencies in vitamins B12 and D may affect the function of TLR2 through increasing TNF-α. To the best of

our knowledge, this study is the first to demonstrate this relation in Egyptian children. Further investigations are required to search a new insight in innate immunity context for illustration of the beneficial effects of vitamins B12 and D.

CONCLUSION

The high vitamin deficiencies found in this study confirm the importance of increased awareness and supplementation of vitamins B12 and D.

CONFLICT OF INTEREST

There was no conflicts of interest exist.

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AUTHOR CONTRIBUTIONS

SRE contributed in the research idea, specimens processing, biochemical analysis and interpretation of results, wrote and revised the manuscript. HS contributed in research idea, specimens processing, biochemical analysis and interpretation of results, statistical analysis and wrote the manuscript. HS who was responsible for correspondence. SS reviewed the manuscript. SMS who was screened the subjects and gave the clinical data. NN contributed in biochemical analysis. MME provided the equipment's for cell culture. HSK who was collected the samples and clinical data. All authors read and approved the final version.

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