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Exploring possible relationships between 25(OH)D deficiency and variables related to inflammation, endothelial function, and enzymatic antioxidants in adolescent athletes: a prospective study

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ABSTRACT: Although the role of vitamin D in calcium and bone metabolism is well documented, there is little clarity regarding the implications of low vitamin D status for inflammation, endothelial function, and antioxidant status in adolescent athletes. A prospective cohort study was conducted, and 44 male adolescent athletes, training at a sports academy in the Middle East, were assigned to either the intervention group (VitDs), consisting of vitamin D deficient athletes [twenty-five hydroxyvitamin D (25(OH)D) <20 ng/ml; n = 22], or the comparison group, consisting of vitamin D sufficient athletes [25(OH)D >30 ng/ml; n = 22]. Vitamin D status, inflammatory cytokines, endothelium-related variables, and antioxidant enzymes were measured twice during a nine-week training period. At the baseline, the athletes in the VitDs group had significantly lower concentrations of 25(OH)D, vascular endothelial growth factor (VEGF), and glutathione peroxidase (GPx), and higher levels of parathyroid hormone (PTH), interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1ra), and nitrite (NO_2) ($p < 0.05$), in comparison to the athletes in the sufficient group. After vitamin D supplementation for the VitDs group, the two cohorts differed considerably in vitamin D binding protein (VDBP) and PTH concentrations ($p < 0.05$). Our data suggest that the low levels of vitamin D possibly induced alterations in the investigated biochemical parameters of athletes in the VitDs group at the beginning of the monitoring period. Furthermore, while the vitamin D supplementation was effective in increasing 25(OH)D status, it may have concurrently positively influenced variables that are related to inflammation, endothelial function, and enzymatic antioxidants.

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INTRODUCTION

Vitamin D plays a vital role in calcium and bone metabolism [11], and its relevance for athletic performance has been adequately detailed in recent literature [6]. In cohorts of athletes, the indication of deficiency varies by season, location, and sport [13]. Moreover, specific populations, such as individuals of Arab origin appear to have an endemic prevalence of low vitamin D concentrations [3, 17, 31]. Correspondingly, it was reported that Arab adolescent students, training at a sports academy in the Middle East, demonstrated a significant prevalence of hypovitaminosis D [25].

In non-athletic populations, studies have detected substantially higher pro-inflammatory cytokine levels in vitamin D deficient adults in comparison to adults with sufficient vitamin D levels [4, 19]. Preliminary results from studies carried out in sports populations present similar findings, regarding the kinetics of pro-inflammatory cytokines [38, 23, 34]. In sports, the role of these cytokines is of high importance because it is hypothesised that they can increase an individual's susceptibility to muscle injury and can play a significant role during overreaching/overtraining syndrome [32, 33]. In addition, under inflammatory conditions, neutrophils become activated and their longevity considerably expands, generating free radicals [36].

Few investigations have associated vitamin D deficiency with endothelial dysfunction [1]. A sufficient 25(OH)D concentration can preserve the endothelial function by increasing the endothelial

cell proliferation, or by enhancing nitric oxide synthase (eNOS) and nitric oxide (NO) generation [15]. In a relatively novel work [39], it was suggested that the active form of vitamin D can affect the up-regulation of VEGF and the superoxide dismutase (SOD) expression in endothelial cells. Vitamin D can also possibly be considered as an antioxidant that protects the endothelium from oxidative stress damage, since it can reduce the generation of anion superoxide [37].

While it is well recognised that vitamin D plays a fundamental role in calcium metabolism and bone health, there is less clarity on the implications of low vitamin D levels in adolescent athletes. Increased inflammation, oxidative stress, and concurrently reduced antioxidants could affect athletes' health, trainability, and performance. Through this prospective study, we aimed to examine potential relationships between vitamin D status and circulating cytokines, antioxidants, and endothelium function-related parameters, and determine the effectiveness of an intervention scheme to correct hypovitaminosis D. Based on the current literature, we hypothesised that athletes with deficient 25(OH)D concentrations should present with an unfavoured redox status profile and increased inflammation markers, which could be corrected by vitamin D supplementation.

MATERIALS AND METHODS

Experimental design

This study was a prospective analysis of a nine-week vitamin D supplementation based on the clinical recommendation. Athletes were screened for vitamin D status and other health aspects at the beginning of February and were assigned to vitamin D supplementation if their 25(OH)D levels were identified to be deficient (<20 ng/ml). Athletes with a sufficient 25(OH)D concentration (>30 ng/ml) were not prescribed vitamin D supplementation. For the purpose of this analysis, we divided the cohort in two groups as follows: the vitamin D supplemented group (VitDs) consisting of 22 vitamin D deficient athletes [25(OH)D concentration range 11.3-19.3 ng/ml] and the comparison group consisting of 22 vitamin D sufficient athletes [25(OH)D concentration range 30.3-69.2 ng/ml]. The screening is part of the periodic medical assessment conducted at the sports academy. The group of athletes identified as vitamin D deficient was provided with 50,000 IU.week^{-1} of vitamin D_3, after their main meal. The rest of the young athletes were not receiving any supplement or placebo. Complete data (first and second measurements) were available for 44 athletes. During the period of the prospective analysis, the athletes continued their typical training activities with no difference between the two groups.

Participants

From a broader pool of athletes, data from 44 adolescent male athletes of Arab origin of a Sports Academy in the Middle East were prospectively analysed based on their vitamin D status. Athletes practised a wide variety of individual sports. In particular, athletes in the vitamin D deficient group trained in athletics (n = 10), fencing (n = 4), gymnastics (n = 2), golf (n = 1), squash (n = 3), shooting (n = 1), and table tennis (n = 1), while athletes in the vitamin D sufficient group trained in athletics (n = 12), fencing (n = 1), gymnastics (n = 2), golf (n = 1), squash (n = 2), shooting (n = 2), and table tennis (n = 2). They typically performed five to nine sessions per week and had been regularly training for six months to five years. The necessary criteria for inclusion were that the athletes were free of identified injuries and were not taking medications two weeks before the data collection. The study was conducted in compliance with the Declaration of Helsinki and approved by the local ethics committee (IRB Approval # 20140000012) and the AZF Research Committee. Parents had signed the informed consent to allow the children to take part in the screening procedures, which were part of a large study on growth and maturation of young athletes.

Anthropometric measurements

Anthropometric and body composition characteristics were measured one day before blood collection [VitDs group: age 14.3 ± 1.7 years, body mass 58.5 ± 21.9 kg, height 163.2 ± 11.1 cm, body mass index (BMI) 21.4 ± 5.4; Sufficient group: age 14.8 ± 1.7 years, body mass 57.8 ± 18.0 kg, height 165.6 ± 14.7 cm, body mass index (BMI) 20.6 ± 4.0 (mean ± *SD*)]. Briefly, body mass was measured with an electronic scale (Marsden, MGP250, UK), while height was determined using a Harpenden Stadiometer (Holtain, UK).

Blood collection and analysis

Blood samples were collected twice during one training season, in February (first measure) and after nine weeks, following an overnight fast. Also, the samples were collected after a training-free day to minimize possible confounding effects caused by exercise. The athletes reported to the laboratory and sat quietly for 15 min before providing a blood sample. Venous blood was collected between 7:30 am and 9:30 am via venipuncture from an antecubital arm vein using a safety butterfly set in BD vacutainer tubes (K2 EDTA) and serum separator tubes (SST 2 Advance, USA). Samples were centrifuged at 1500 *g* for 15 min to separate plasma and serum and stored at -80°C until further analysis.

Laboratory analyses

In serum, VDBP, C-reactive protein (C-RP), IL-6, IL-1ra, NO_2, and total nitrite/ nitrate ratio (NO_2/NO_3) were assessed using ELISA kits from R&D diagnostics (R&D, International Inc., USA). The mean intra-assay coefficient of variation (CV) was 5.9%, 6.0%, 7.1%, 5.5%, and 1.9%, respectively, while their inter-assay CV was less than 9.6%. PTH and 25(OH)D concentrations were also analysed using ELISA kits from BioVendor (Brno, Czech Republic) and EAGLE Bioscience (Nashua, NH, USA), correspondingly. The intra- and inter-assay CV were less than 2% and 7.1% for PTH, and less than 5.0% and 11% for 25(OH)D, respectively. In plasma, VEGF and SOD were assessed using ELISA kits from Abcam (Cambridge, UK), while CAT and GPx were analysed with Blue Gene (Shanghai, China). The mean

intra-assay CV for these parameters was 10.0%, 4.1%, 10.0%, and 10.0%, respectively, while their inter-assay was less than 11%.

Statistical analyses

Data were checked for normality before all statistical analyses with a Kolmogorov-Smirnov test. At baseline, an independent *t*-test was applied for the exploration of differences between the two groups after clustering according to 25(OH)D levels. As the two groups significantly diverged at the baseline, an analysis of covariance (ANCOVA) was performed for the investigation whether the post-test means, adjusted for pre-test scores, age and BMI, differ between the two groups. A paired *t*-test was performed for the identification of the within-groups changes. Effect sizes (ES) were determined by calculating partial eta squared (η^2) and Cohen's *d*. According to Cohen [10], the magnitude of ES (η^2) can be classified as small ($0.01 \leq \eta^2 < 0.06$), medium ($0.06 \leq \eta^2 < 0.14$), and large ($\eta^2 \geq 0.14$), while the magnitude of Cohen's *d* can be classified as trivial (<0.2), small (0.21-0.60), moderate (0.61-1.20), large (1.21-1.99), and very large (>2.0). The statistical package SPSS (version 18.0) was used for all analyses. The level of significance was set at $p < 0.05$.

RESULTS

Table 1 shows results of variables related to vitamin D status and inflammation of the two examined groups, before and after a nine-week period, mean ± *SD* (SD). At baseline, the comparison group displayed higher 25(OH)D (large ES) and lower PTH, IL-6 and IL-1ra concentrations (moderate ES) in comparison to the VitDs group ($p < 0.05$). After vitamin D supplementation for the vitamin D deficient group, the two cohorts differed considerably in VDBP and PTH (large ES size; $p < 0.05$).

Table 2 presents results of endothelium-related variables and endogenous antioxidant enzymes of the two examined groups, before and after a nine-week period, mean ± SD (SD). Before supplementation, the VitDs cohort displayed lower GPx, VEGF and NO_2 concentrations (moderate ES) compared to the sufficient group ($p < 0.05$). These differences were not evident after the supplement intervention.

The analysis of within-group variations confirmed the expected increase in 25(OH)D concentration in the VitDs group, in parallel to an increase in VDBP and VEGF concentrations ($p < 0.01$) and reduced PTH levels ($p < 0.05$) after the vitamin D supplementation. Also, in the vitamin D sufficient group, a significant increase in 25(OH)D ($p < 0.01$) and SOD ($p < 0.05$) concentrations and a decrease in VDBP and PTH levels ($p < 0.01$) were observed at the follow-up screening.

DISCUSSION

One of the aims of the current research was to monitor the effectiveness of a nine-week supplementation programme on a vitamin D deficient athletic group of Arab origin. The recommended dosage by the Academy's medical team reported for this study can be

TABLE 1. Variables related to vitamin D status (PTH, VDBP) and inflammation (IL-6, IL-1ra, C-RP) of the two examined groups (vitamin D deficient (VitDs) and sufficient group, mean ± *SD*).

Variables	Testing	Deficient	Sufficient	*p*	Cohen' d	η^2
25(OH)D [ng/ml]	Pre	16.0 ± 2.6	36.7 ± 10.6$^{\#}$	0.001	2.68	
	Post	46.3 ± 14.8**	55.8 ± 16.5**	*0.203*		0.041
VDBP [ug/ml]	Pre	156.4 ± 53.9	195.7 ± 63.3	*0.050*	0.66	
	Post	188.4 ± 38.8**	149.3 ± 62.8$^{\$**}$	0.001		0.339
PTH [pg/ml]	Pre	80.0 ± 49.6	46.9 ± 15.4$^{\#}$	0.010	0.90	
	Post	48.9 ± 27.6*	29.3 ± 13.0$^{\$**}$	0.013		0.177
IL-6 [pg/ml]	Pre	0.96 ± 0.35	0.72 ± 0.30$^{\#}$	0.022	0.73	
	Post	1.15 ± 0.60	0.90 ± 0.50	*0.727*		0.003
IL-1ra [pg/ml]	Pre	382.1 ± 189.1	271.5 ± 95.0$^{\#}$	0.019	0.73	
	Post	335.9 ± 153.2	266.5 ± 76.7	*0.371*		0.021
C-RP [mg/l]	Pre	0.82 ± 1.13	0.46 ± 0.45	*0.165*	0.41	
	Post	0.91 ± 0.84	0.58 ± 0.49	*0.315*		0.026

Note: $^{\#}$Significant differences between groups at baseline, $^{\$}$Significant differences between groups after supplementation, *and**=Significant differences within groups for $p \leq 0.05$ and $p \leq 0.01$, respectively. Abbreviations: 25(OH)D=twenty-five hydroxyvitamin D, VDBP=vitamin D binding protein, PTH=parathyroid hormone, IL-6=interleukin-6, IL-1ra=interleukin-1 receptor antagonist, C-RP=C-reactive protein.

TABLE 2. Variables related to **e**ndothelial function (VEGF, NO_2, NO_2/NO_3) and endogenous antioxidant enzymes (SOD, CAT, GPx) of the two examined groups (vitamin D deficient (VitDs) and sufficient group, mean ± *SD*).

Variables	Testing	Deficient	Sufficient	*p*	Cohen' d	η^2
SOD [ug/ml]	Pre	38.2 ± 10.1	36.7 ± 16.4	*0.711*	0.13	0.023
	Post	42.3 ± 10.9	45.6 ± 11.8*	*0.340*		
GPx [ug/ml]	Pre	24.3 ± 5.9	29.4 ± 9.4$^{\#}$	*0.038*	0.64	0.001
	Post	25.2 ± 8.8	27.8 ± 8.1	*0.860*		
CAT [ug/ml]	Pre	17.7 ± 20.4	23.8 ± 22.3	*0.353*	0.28	0.013
	Post	17.4 ± 23.6	22.5 ± 24.1	*0.480*		
VEGF [pg/ml]	Pre	184.6 ± 85.6	263.9 ± 149.2$^{\#}$	*0.036*	0.65	0.040
	Post	267.8 ± 144.3*	309.2 ± 165.2	*0.208*		
NO_2 [umol/l]	Pre	72.2 ± 26.5	108.5 ± 68.7$^{\#}$	*0.026*	0.67	0.001
	Post	81.5 ± 43.6	90.0 ± 38.6	*0.992*		
NO_2/NO_3 [umol/l]	Pre	229.6 ± 77.4	215.7 ± 63.7	*0.520*	0.19	0.023
	Post	206.0 ± 67.4	223.4 ± 53.6	*0.342*		

Note: $^{\#}$Significant differences between groups at baseline, $^{\$}$Significant differences between groups after supplementation. *and**=Significant differences within groups for $p \leq 0.05$ and $p \leq 0.01$, respectively. Abbreviations: SOD=superoxide dismutase, GPx=glutathione peroxidase, CAT=catalase, VEGF=vascular endothelial growth factor, NO_2=endogenous nitrite, NO_2/NO_3=nitrite/nitrate.

characterised as high when compared to previous prescriptions. The US Institute of Medicine (IoM) has set a tolerable upper intake of 4,000 IU per day for young adults [22]. In many publications involving athletes, higher dosages of vitamin D supplementation have been implemented. Depending on the threshold that must be attained, supplementations of 5,000 or 10,000 IU per day are cited in various studies that include athletes [8].

Moreover, 10,000 IU per day has been proposed as a safe, tolerable upper intake level [18], and a single weekly dose of 50,000 IU for eight weeks is frequently used to improve deficient statuses [21]. However, a recent study aimed to identify the responses of all critical vitamin D metabolites to moderate and high dose supplemental vitamin D_3, i.e., 35,000 or 70,000 IU per week. The results determined that both schemes improved the participants' vitamin D status, but the higher dose resulted in a significant increase of the metabolite $24,25[OH]_2D$, and a decrease of the bioactive form of vitamin D [26]. In any case, the intervention was successful in increasing the levels of 25(OH)D in the VitDs cohort up to more acceptable levels (>30 ng/ml).

In the February baseline assessment conducted in this study, the two cohorts both increased their 25(OH)D levels; however, no statistically significant difference was evident between the groups after the VitDs group had completed the supplement intervention. Also, both groups varied considerably in PTH levels at both time points (with a moderate and large ES, respectively), and in VDBP during the follow-up screening (large ES). In the VitDs group, PTH levels followed a reversal to 25(OH)D kinetic (i.e., when vitamin D was low, PTH was high, and vice versa). The studies of Holick et al. [20, 21] concur that low vitamin D concentrations trigger the calcium sensor to increase PTH production, which usually begins to plateau as blood vitamin D levels range between 30 and 40 ng/ml. Furthermore, although the examined groups significantly differed in VDBP levels after the intervention, this variable is considerably raised in the VitDs group and decreased in the comparison group. In principle, VDBP protects vitamin D from biodegradation, and facilitates its transport to tissue targets, but its levels do not influence the effect of vitamin D repletion on serum PTH and calcium [16, 29]. In addition, distinctive single-nucleotide polymorphisms (SNPs) are recognised to modulate VDBP levels and affinity for 25(OH)D [3] and provide insight into why specific ethnic groups may have a particular 25(OH)D and BDM relationship [2,30].

The alternative implications of vitamin D, principally those associated with cytokines, endothelial function, and redox status, have not been studied extensively in young athletic populations. Few investigators have supported the theory that inflammation reduces 25(OH)D concentration, while others defend the opposite view, with the evidence suggesting the latter perception [7]. The present study revealed that the two groups significantly diverged regarding their IL-6 and IL-1ra concentrations at the initial measure, with the VitDs athletes revealing higher levels of both cytokines. However, these distinctions disappeared after the VitDs group completed the supplement intervention. It seems that the vitamin D administration might have had an impact on these cytokines.

In sports, the moderate increase of particular pro-inflammatory cytokines for a prolonged period induces a "chronic low-grade" inflammation, which is related to an impairment in performance and

an increased susceptibility to muscle injuries [28, 33]. IL-6 significantly rises after strenuous exercise and it is also a potent inducer of IL-1ra production. Furthermore, IL-1ra inhibits the signal transduction of the pro-inflammatory IL-1, and also acts as an acute-phase protein that increases or decreases in response to inflammation, to regulate diverse physiological systems [33, 27]. Baseline data of the current study show that IL-6 concentration was lower in our cohort compared to adolescent wrestlers and volleyball athletes, while the IL-1ra levels of the VitDs group were significantly higher compared to those athletes [12]. As regards the other inflammation marker, C-RP was not affected in the VitDs group throughout the nine-week period, but always stayed below the reference range for healthy individuals (<1 mg/L) [35].

In concurrence with our findings, Codoner et al. [9]. documented higher IL-6 concentrations in obese adolescents with a vitamin D status below 20 ng/ml, when compared to those with a level above this cut-off. Moreover, Bellia et al. [5] observed a noteworthy relationship between the mean serum 25(OH)D concentration of 65 nmol/L and several inflammatory markers (C-RP, IL-6, and TNF-a) in obese participants. Nevertheless, as the CV of specific assays was rather high and the identification of adolescent reference ranges challenging, the ES of differences can contribute to the interpretation of results. At baseline, the ES between the cohorts was moderate for IL-6 and IL-1ra, and small for C-RP, while at the second screening, the ES was small for these variables.

During long-term aerobic exercise, increased blood flow, NO_2 generation, and VEGF generation support the endothelial function [24]. In our investigation, at the baseline both endothelial-related variables were lower (moderate ES) in the VitDs group as compared to the comparison group. Consequently, this information could convey deterioration of endothelial function in the VitDs cohort of athletes and, in contrast, increased bioavailability of these variables in the vitamin D sufficient group. However, following the supplement intervention, no discrepancies were observed between the two cohorts. Moreover, the assessed groups did not differ in the accumulation of serum SOD and CAT enzymes at the baseline, but they did deviate in GPx levels (moderate ES), which were lower in the VitDs athletes. As the GPx enzyme is fighting higher loads of hydroxyl peroxide free radicals as compared to CAT [14], we can hypothesise that the VitDs group experienced a slightly higher oxidative stress state in the same period. It is well recognised that when the antioxidant system fails to diminish excess reactive oxygen and nitrogen species (RONS), these species react with lipids, proteins, and nucleic acids, forming undesirable modifications [1]. Based on these preliminary results, remedies through vitamin D supplementation programmes should also consider its potential effect on biomarkers linked to inflammation, antioxidant enzymes, and endothelial function.

The results presented in this study have certain limitations. Although we aimed to explore our scope thoroughly, we could not provide more information concerning athletes' diet, injuries, or symptoms of overreaching. Also, the cohort was limited and so was the length of the period of observation.

CONCLUSIONS

The vitamin D supplementation allowed the cohort of vitamin D deficient athletes to recover to acceptable values. However, before the particular supplementation programme, the VitDs athletes showed higher IL-6 and IL-1ra concentrations and lower GPx, VEGF, and NO_2 levels when compared to the sufficient cohort. Based on these preliminary results, it is necessary to conduct larger and more controlled studies to understand the links between vitamin D status and biomarkers linked to inflammation, antioxidant enzymes, and endothelial function in order to ascertain whether vitamin D deficient athletes present increased risks of illness during heavy training periods and/or whether different vitamin D supplementation schemes can reduce such risks.

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