

The supplementary effects of omega-3 fatty acid alone and in a combination with vitamin D3 on serum leptin levels: A randomized clinical trial on men and women with vitamin D deficiency

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Abstract

Purpose: This randomized clinical trial (RCT) was designed to assess the effect of VD₃, n-3FA, and their combination on serum leptin levels in people with vitamin D deficiency (VDD).

Subjects and methods: One hundred and forty six participants, were randomly assigned into four groups supplemented with the dose of 50,000 IU VD₃ taken weekly (D), 300 mg n-3FA taken daily (Om), and their combination (D+Om) or control (C) for eight weeks. Fasting baseline and follow-up (10 weeks; 8 weeks supplementation plus washout period of 2 weeks) of serum 25 hydroxyvitamin D (25OHD), leptin, glucose, triglycerides (TG), parathyroid hormone (PTH), calcium, and phosphorus were assayed. A paired T-test was used to assess the changes in serum leptin levels over of the follow-up period.

Results: Significant increase in follow-up serum leptin (10.62 ± 7.18 to 14.42 ± 8.29 ng/mL, $P = 0.002$) and TG (154 ± 84.4 to 200.1 ± 79 , $P = 0.015$) levels were observed in n-3-FA supplemented group. Combination therapy (VD₃ plus n-3 FA) significantly increased serum 25OHD (13.49 ± 4.64 to 37.09 ± 11.13 ng/mL, $P < 0.001$), TG levels (114.3 ± 57.3 to 139.1 ± 60.7 mg/mL, $P = 0.007$) and insignificantly serum leptin (6.74 ± 4.87 to 8.01 ± 6.77 ng/mL, $P = 0.269$).

Conclusion: Our study referred that notable elevation in leptin and TG levels might be linked to leptin resistance. However, further RCTs are required to clarify possible consequences resulted from the extensive administration of n-3FA supplements and their combinations with high doses of VD₃ supplements on humans' health.

Keywords

leptin, n-3FA, omega, vitamin D₃, diabetes

Introduction

Vitamin D deficiency (VDD), obesity, and diabetes mellitus (DM) are worldwide problems. There has been growing interest in the association of type 2 DM (T2DM) with VDD to obesity (Smith and Singleton 2013). Several studies (Abu-Hasheesh et al. 2010; Al-Amer et al. 2015; Abu-Samak et al. 2019) have demonstrated a high prevalence of T2DM and VDD in Mediterranean countries including Jordan. In this context, Kim et al. (2013) found out that VDD was more prevalent in obese people with inverse association with 25-hydroxy vitamin D (25OHD). On the other hand, several randomized clinical trials (RCTs) (Duggan et al. 2014; Mousa et al. 2020) pointed to a vague association between obesity marker (leptin hormone) and VDD. Centrally, leptin overrides satiety as well as peripherally enhances insulin sensitivity (Lanzerstorfer P et al. 2015). Leptin has anti-diabetogenic effects either via improving insulin resistance (IR) and/or stimulating insulin secretion (D'Souza A et al. 2014). It is classified as an adipokine involved in the IR pathogenesis and development of T2DM (Finucane et al. 2009). Some RCTs (Upreti et al. 2018; Niroomand et al. 2019) showed that the treatment of VDD by VD_3 may improve the control of diabetes or decrease the risk of disease. Nevertheless, RCTs that examined VD_3 supplements on serum leptin levels were contradictory.

According to Dinca et al. (2016), six RCTs, with different therapy protocols, did not show any effect for VD_3 supplements on leptin concentration according to meta-analysis data T2DM. For example, Duggan et al. (2014) showed that the effect of a small dose of VD_3 (2000 IU/day) for 12 months did not change serum leptin. Furthermore, a recent RCT (Mousa et al. 2020) with a higher dose (100,000 IU single bolus followed by 4,000 IU daily) for 16 weeks, showed no differences in leptin levels. Conversely, Hajimohammadi et al. (2017) mentioned a positive association between leptin and 25OHD levels. Elevated serum leptin was also observed after VD_3 intervention conducted on 64 patients with the last stage of kidney failure T2DM (Naini AE et al. 2016). In turn, leptin levels were significantly decreased after a single dose of VD_3 (600,000 IU) in 12 subjects with extreme obesity ($BMI > 40 \text{ kg/m}^2$) T2DM (Mai S et al. 2017). Based on the above, despite the consensus of several observational studies (Kim et al. 2013; Turer et al. 2013) that linked VDD to obesity, RCTs did not confirm the effects of VD_3 supplementations on serum leptin levels as a key link to insulin resistance of T2DM.

On the other hand, conflicting data obtained from RCTs showed that there are no (Poreba et al. 2017; Sedlacek et al. 2019) or insignificant reduction (Hariri et al. 2015) in serum leptin after n-3FA intervention. Farimani et al. (2018) showed similar findings in diabetic patients. Generally, the effects of n-3FA on serum leptin in obese people or diabetics are still unclear and need more clarifications according to many systematic reviews. In the same context, a potential synergistic association between VD_3 and n-3FA supplementations has taken increase attention in the recent clinical trials (Manson et al. 2015; Al-

Shaer et al. 2019; Pittas et al. 2019). Nevertheless, despite the two supplements are widely recommended for several therapeutic aims, studies that highlight the effects of such combination are still not enough. Cadario et al. (2017) administrated the co-supplementation to sustain the remission of T1DM. It reduced the number of wet nights among 7–15-year-old children with nocturnal enuresis according to Rahmani et al. (2018). Razavi et al. (2017) also pointed that VD_3 and n-3FA co-supplementation for 6 weeks had beneficial effects in gestational diabetes mellitus.

Conversely, a recent RCT (Pittas et al. 2019) among a total of 2432 persons, at high risk for T2DM, found out that VD_3 supplementation at a dose of 4,000 IU per day did not lower the risk of diabetes. Accordingly, this RCT was designed to assess the co-supplementation effects of VD_3 50,000 IU once weekly and n-3FA 300 mg per day on serum leptin in men and women with VDD.

Experimental part

Materials and methods

The current randomized clinical trial (RCT) was authorized by the Institutional Review Board (IRB) committee of Applied Science Private University (ASU) (protocol no. DRGS-4-2018-1) and conducted during the winter season, between December 2018 and March 2019. The RCT was performed according to the declaration of Helsinki. The informed consent was filled from each enrolled participant in this clinical trial. Participants were Jordanian men and women from the ASU community in addition to ASU employees' relatives with an average age at baseline of the trial was 36.18 ± 9.74 years (range 25 to 55). Eligible participants were enrolled based on a confirmed diagnosis of 25OHD levels deficiency (VDD) by internal medicine consultants in Ibn Al-Haytham hospital laboratories. People previously diagnosed with chronic diseases such as kidney problems were excluded due to the association between prolonged administration of VD_3 and kidney stone formation (Jackson et al. 2006). Exclusion criteria from the trial also included participants diagnosed with chronic diseases such as osteoporosis, cancer, endocrine disorder and, thalassemia or who have a documented history of allergic reactions to n-3FA supplementations.

Intervention

The baseline and follow-up measurements of the anthropometric and clinical variables were recorded before and after VD_3 and n-3FA supplementation. At the end of the interventional protocol which is 8 weeks, the participants entered a washout period of 2 weeks. Then, follow-up measurements were collected for all the participants. A computer-generated randomization was created by an independent statistician. Eligible participants ($n = 188$) were allocated into four groups as illustrated in the consort chart (Figure 1): Group 1 (C); Participants received no treatment and served as the control group; Group 2

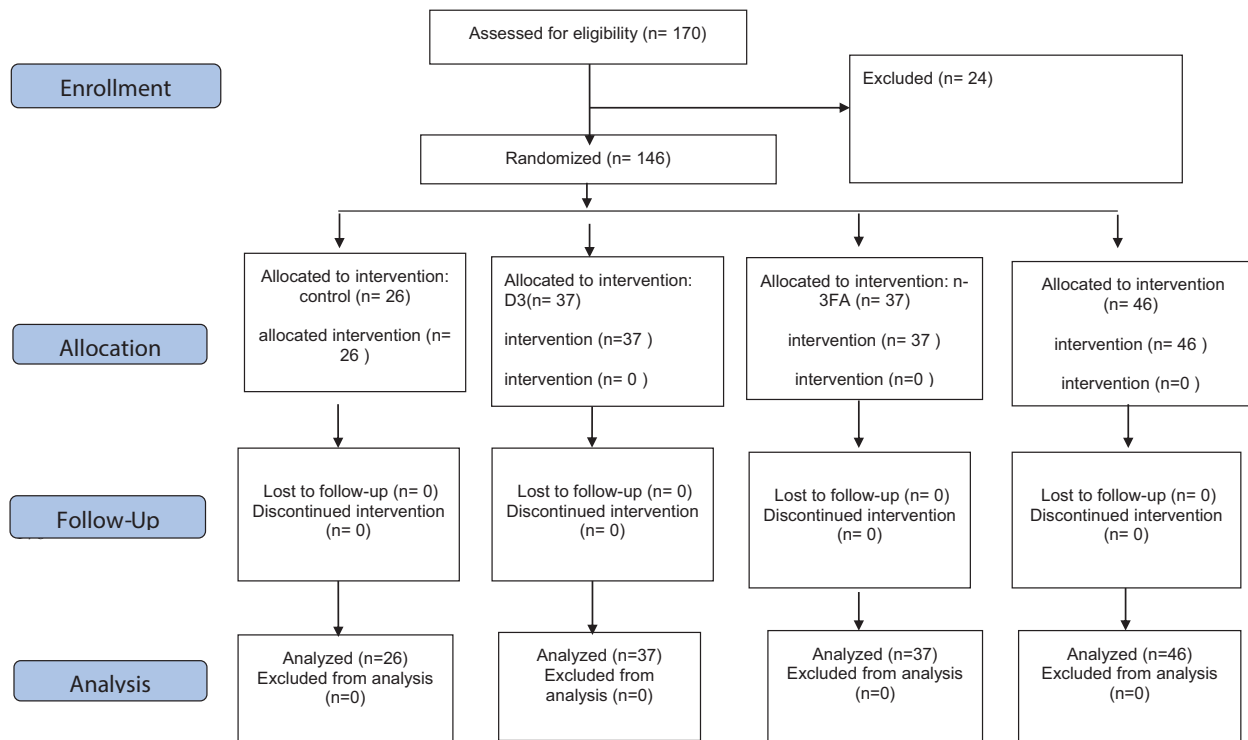


Figure 1. CONSORT flow diagram for the study, indicating the number of subjects screened, recruited, and randomly assigned to the different intervention groups.

(D); Participants received 50,000 IU of VD₃ in a Hi Dee soft gelatin capsule (United Pharmaceuticals Company (Amman, Jordan) once weekly; Group 3 (Om); Participants received soft gelatin capsule of 1,000 mg of wild salmon and fish oil complex (Jamieson Laboratories., Toronto, ON, Canada) once daily. Each capsule contains 300 mg of omega3-FA, equivalent to 300 mg of n-3FA (180 mg as eicosapentaenoic acid and 120 mg as docosahexaenoic acid [DHA]). Group 4 (D+Om); Participants treated with 50,000 IU VD₃ once a week and 1000 mg wild salmon and fish oil complex (contains 300 mg of Ω -3FA) once daily. D+Om participants were advised to leave at least a 4 to 6-hour time interval was followed between administering the two supplements. Therapeutic protocols of VD₃ and n-3FA supplements were approved in accordance with the Endocrine Society's Clinical Guidelines for treating VDD in adults (Holick et al. 2011). A Similar protocol of VD₃ over 12 months was conducted on humans and did not produce any toxicity (Binkley et al. 2011). DHA doses used in many previous trials were also comparable to the dose used in this trial protocol without side effects as reported in children taking a dose of 600 mg DHA/day (Richardson et al. 2012). Patients' adherence to the therapeutic protocol was followed via regular mobile phone text messages.

Anthropometric measurement

This RCT was conducted at Pharmacy school laboratories/ ASU during the winter of 2018 where the timing of blood sampling is important to minimize seasonal fluctuations of total levels of vitamin D in the blood (Wacker

M and Holick MF 2013). Anthropometric parameters including height (Ht), body weight (BW), body mass index (BMI), waist (W) circumference, hip (H) circumference, waist/hip ratio (WHR) were measured at baseline and end of the trial.

Clinical parameters assays: After collection, into labeled Eppendorf tubes, serum measurement of clinical parameters was done at the clinical laboratories of Ibn Al-Haytham hospital Amman, Jordan. **Vitamin D:** Chemiluminescence immunoassay LIAISON 25-hydroxyvitamin D Assay (DiaSorin) was used to measure a total serum concentration of vitamin D (25OHD). The lower limit of assay approximately was (4 ng/mL) with a 100% cross-reactivity with both metabolites of 25OHD, 25OHD₂, and 25OHD₃ and thus measures total serum 25OHD content. **Leptin:** Serum levels of leptin were assayed using the enzyme Immunoassay kit (leptin EIA -5302, DRG Diagnostics, Marburg, Germany). Assay Analytical sensitivity was 0.1 ng/mL. **Parathyroid Hormone:** Serum levels of parathyroid hormone (PTH) were assayed using the enzyme Immunoassay kit (PTH Intact EIA -3645, DRG Diagnostics, Marburg, Germany). The assay analytical sensitivity of the test was 1.57 pg/mL. **Calcium and Phosphorus:** Calcium and phosphorus (PO₄) concentrations in serum were assayed by spectrophotometry method (Clinical Chemistry RAL Analyzers Clima Plus, Spain) using (CALCIUM-ARSENAZO kit (M11570i-15) and Phosphorus Phosphomolybdate/Uv Kit (M11508i-18, BioSystems, Spain). **Glucose and Triglycerides:** Serum fasting blood glucose (FBG) and triglyceride (TG) levels were assayed on a Roche Cobas C501 analyzer (GLUC3 application, Roche, Mann-

heim, Germany) using enzymatic colorimetric method. Serum TG levels were measured using TG -BioSystems kits (M11528i-20, BioSystems, Barcelona, Spain). **Alanine Aminotransferase (ALT)** and **Aspartate Aminotransferase (AST)** were measured using specific ALT and AST BioSystems kits (M21533i-03, M11531i-23, Barcelona, Spain). **Serum creatinine (Cr)** and **urea** levels were measured using Creatinine kit (M12502i-19) and Urea (M11536i-17) kit, BioSystems, Barcelona, Spain) with detection limits = 0.04 mg/dL and 1.3 mg/dL respectively.

Statistical analysis

The parameter changes at the follow-up period (10 weeks) within each group were assessed by paired Student's *t*-test. To investigate if there is any significant difference in the mean values for each parameter between the different groups of trial One Way ANOVA test was used. Post hoc comparisons using the Tukey HSD test were conducted to provide specific analysis on which means are significantly different from each other in the four trial groups, differences were considered significant at $P < 0.05$. To determine potential factors that influenced leptin at the baseline-end of the trial, multiple linear regression was applied using the stepwise method according to the different groups. Kolmogorov-Smirnov test was used to evaluate the normal distribution of study parameters. Data was approximately close to the normal distribution because of the large sample size of the trial. These statistical analyses were performed using SPSS a Statistical Package for the Social Sciences.

Results

The consort diagram (Figure 1) shows that one hundred and forty-six participants completed all stages of the trial out of one hundred seventy eligible participants. Approximately sixty percent of the participants were females ($n=88$). Due to the poor medication adherence or complying with blood sampling instructions, there were twenty four participants were dropped out of the study. The parental history of DM showed that 18.5% of the participant's fathers had DM history whereas 24% of the participant's mothers were diabetic. Furthermore, 49% of the participants practice morning sun exposure.

Baseline participants' characteristics

The mean age of all participants at baseline of the trial was 36.18 ± 9.74 years (Table 1). BMI mean was 27.57 ± 5.38 kg/m² which indicated that the population of the study sample was generally overweight. The mean value of 25OHD for all the trial participants was 16.43 ± 7.34 ng/ml which means they were VD deficient. Any volunteer with a vitamin level equals to or higher than 30 ng/mL was not recruited to participate in this RCT. Descriptive analysis of the mean values for clinical parameters is presented in Table 2, all values were within normal ranges.

Table 1. Baseline Descriptive Statistics of the Anthropometric Parameters ($n = 146$).

Anthropometric Parameter	Mean \pm SD	Range
Age (year)	36.18 \pm 9.74	25.00-55.00
Weight (kg)	77.47 \pm 16.11	42.00-128.00
Height (cm)	166.33 \pm 7.93	148.00-187.00
BMI (kg.m ⁻²)	27.57 \pm 5.38	14.48-50.08
Waist (cm)	92.01 \pm 14.57	46.00-139.00
Hip (cm)	106.77 \pm 12.56	43.00-158.00

Note: SD: Standard deviation; BMI: body mass index.

Table 2. Baseline Descriptive Statistics of the Clinical Parameters ($n = 146$).

Clinical parameter	Mean \pm SD	Range	Normal ranges
25OHD (ng/mL)	16.43 \pm 7.34	3.80-28.2	30-50
FBG (mg/dL)	84.88 \pm 17.79	67.10-115.20	70-110
Leptin (ng/mL)	8.78 \pm 6.44	1.10-31.70	*NA
TG (mg/dL)	129.61 \pm 67.12	26.30-401.50	Up to 150
PTH (pg/mL)	33.56 \pm 10.95	9.26-50.00	9-90
Calcium (mg/dL)	9.86 \pm 1.78	8.50-18.60	8.6-10.3
PO ₄ (mg/dL)	4.12 \pm 0.27	3.30-5.10	2.5-4.5
Cr (mg/dL)	0.90 \pm 0.07	0.50-1.20	0.5-1.1
ALT (U/L)	9.36 \pm 3.43	0.90-33.00	Up to 65
AST (U/L)	7.29 \pm 1.72	3.00-18.00	Up to 30
Urea (mmol/L)	3.03 \pm 0.73	0.90-7.50	2.5 to 7.1

Note: 25OHD: 25-hydroxy vitamin D; FBG: fasting blood glucose; TG: triglycerides; PTH: parathyroid hormone; PO₄: phosphate; Cr: creatinine; ALT: alanine aminotransferase; AST: aspartate aminotransferase. * NA, not applicable.

Correlation of selected obesity variables with 25OHD levels

A significant negative correlation between 25OHD and leptin levels was observed in people with VDD at the baseline of the trial ($R = -0.257$, $P = 0.002$). A negative correlation but insignificant was also observed between 25OHD levels and BMI values as shown in Table 3 ($R = -0.153$, $P = 0.066$). No significant correlations for the mentioned variables with 25OHD were observed at end of the trial.

Table 3. Correlation of selected obesity variables with 25OHD levels at baseline and follow-up.

Obesity variable	Baseline		Follow up	
	R	P-value	R	P-value
BMI (kg.m ⁻²)	-0.153	0.066	*-0.032	0.705
Weight (kg)	-0.003	0.975	**0.112	0.179
Waist (cm)	-0.062	0.458	0.072	0.391
Leptin (ng/mL)	-0.257	0.002	-0.078	0.347
WHR	0.119	0.154	-0.088	0.291

Note: *: negative correlation, **: positive correlation

BMI: body mass index; WHR: waist/hip ratio; R: correlation coefficient.

Follow-up results

25OHD levels

At the end of the trial, significant differences were observed between the baseline and follow-up mean serum 25OHD levels in the three intervention groups (P -value < 0.01) as shown in Table 4. The mean levels of serum 25OHD were significantly increased ($P^A < 0.01$) in D (38.4 ± 13.4 vs 17.42 ± 5.7 ng/mL) as well as in D+Om study groups (39.7 ± 11.8 vs 16.95 ± 4.8 ng/mL). On the other hand, a signifi-

Table 4. Fasting serum 25OHD at baseline and follow-up.

Group	Fasting serum 25OHD (ng/mL)				P-value
	C	D	Om	D+Om	
Baseline	18.34 ± 7.2	17.42 ± 5.7	19.7 ± 6.03	16.95 ± 4.8	$P^B = 0.1$
Follow-up	17.7 ± 6.5	38.4 ± 13.4	12.34 ± 5.9	39.7 ± 11.8	$P^C < 0.001$
Change	0.65	-20.95	7.38	-22.75	
P^A	0.26	< 0.01	< 0.01	< 0.01	

Note: Change = baseline mean – follow-up mean

P^A : P-value for paired *t*-test between baseline and the follow-up of the study for each group; P^B : P-value for ANOVA with post hoc Tukey HSD done at baseline to compare among all groups; P^C : P-value for ANOVA with post hoc Tukey HSD (honestly significant difference) done at the follow-up of the study to compare among all groups.

Abbreviations: 25OHD: 25-hydroxy vitamin D; C: control group; D: vitamin D3 supplementation group; Om: n-3FA supplementation group; D+Om: vitamin D3 plus n-3FA supplementation group.

cant decrease in 25OHD levels was observed in Om study group ((12.34 ± 5.9 vs 19.7 ± 6.03 ng/ml, $P^A < 0.01$).

Serum leptin levels

Exceptionally, only the Om group (Table 5) showed a significant increase ($P = 0.002$) in the mean serum leptin levels at the end of the trial compared to baseline (10.62 ± 7.18 ng/mL vs 14.42 ± 8.29 ng/mL). Post-hoc test at follow-up (Suppl. material 1: Table S1) also indicated that the mean levels of leptin in the Om group were significantly increased in comparison with the control group.

Table 5. Serum levels of leptin at baseline and follow-up.

Group	Serum leptin ng/ml				P-value
	C	D	Om	D+Om	
Baseline	10.11±8.5	8.54±5.1	10.62±7.2	7.74±4.9	$P^B = 0.083$
Follow-up	11.49 ± 10.3	10.26±7.6	14.42±8.3	8.01±6.8	$P^C = 0.005$
Change	-1.38	-1.72	-3.81	-0.27	
P^A	0.501	0.083	0.002	0.269	

Note: Change = baseline mean – follow-up mean

C: control group; D: vitamin D3 supplementation group; Om: n-3FA supplementation group; D+Om: vitamin D3 plus n-3FA supplementation group.

P^A : P-value for paired *t*-test between baseline and the follow-up of the study for each group; P^B : P-value for ANOVA with post hoc Tukey HSD (honestly significant difference) done at baseline to compare among all groups; P^C : P-value for ANOVA with post hoc Tukey HSD done at the follow-up of the study to compare among all groups.

Serum PTH, calcium, and phosphate levels

In the current RCT, compared to baseline levels, no significant changes were noted in serum PTH and calcium at follow-up. In turn, except for the C group, phosphorus levels showed notable changes in all study groups with a significant decrease in the means of serum phosphorus (Suppl. material 1: Table S2).

Serum TG levels

The mean serum TG levels were significantly increased in both Om and D+Om groups (P -value = 0.015, 0.007, respectively) as shown in Table 6.

Stepwise regression analysis

The multivariate stepwise regression analysis showed that the predictors at the period of follow-up, that are included in the model together explained ~50%, 33%, 13%, and 49% of the variance in leptin levels in the C, D, Om, and D+Om study groups, respectively (Table 7).

Table 6. Serum TG levels at Baseline and at End of the Trial.

Group	Control	D	Om	D+Om	p-Value
TG Baseline	120.1±48.6	130.9±65.5	154.0±84.4	114.3±57.3	$P^B = 0.058$
Follow-up	113.4±53.8	137.3±85.3	200.1±79	139.1±60.7	$P^C < 0.05$
Change	6.7	-6.4	-46.1	-24.8	
P^A	0.328	0.608	0.015	0.007	

Note: Change = baseline mean – follow-up mean

C: control group; D: vitamin D3 supplementation group; Om: n-3FA supplementation group; D+Om: vitamin D3 plus n-3FA supplementation group; TG: Triglycerides.

P^A : P-value for paired *t*-test between baseline and the follow-up of the study for each group; P^B : P-value for ANOVA with post hoc Tukey HSD (honestly significant difference) done at baseline to compare among all groups; P^C : P-value for ANOVA with post hoc Tukey HSD done at the follow-up of the study to compare among all groups.

Table 7. The Multivariate Association between the Trial Variables and Serum Levels of Leptin Using Stepwise Regression at the period of the follow-up.

Dependent variable	Study group	Univariate effect estimates	Coefficient			
			B	F	R	R ²
Leptin (ng/mL)	C	Calcium (mg/dL)	**3.157	14.245	0.610	0.372
		ALT (U/L)	1.741	11.510	0.707	0.500
	D	Paternal DM	*-5.433	10.712	0.484	0.234
		Weight (kg)	-0.125	8.312	0.573	0.328
	Om	AST (U/L)	1.677	5.088	0.356	0.127
		D+Om	Calcium (mg/dL)	1.434	12.476	0.470
	PTH (pg/mL)		0.123	10.295	0.569	0.324
	Height (cm)		-0.199	8.989	0.625	0.391
	Gender		-3.841	8.55	0.674	0.455
	Sun-exposure	0.229	15.336	0.685	0.469	
	HtWt R (cm/Kg)	0.005	13.510	0.701	0.491	

Note: *: negative correlation, **: positive correlation

C: control group; D: vitamin D3 supplementation group; Om: n-3FA supplementation group; D+Om: vitamin D3 plus n-3FA supplementation group; ALT: alanine aminotransferase; DM: Diabetes Mellitus.

Discussion

This novel clinical trial on people with VDD indicated that 300 mg daily for 8 weeks of n-3FA significantly raised serum leptin levels concurrently with the decrease of the 25OHD levels. Our observations are in agreement with previous RCTs (Gray et al. 2013; Huang et al. 2019) showed a positive association between leptin and n-3FA levels in obese participants. Consistent with the results of a previous study (Forsythe et al. 2012), baseline findings of the current RCT indicated an inverse association between VDD and serum leptin. It was proposed that adipose tissue absorbs the fat-soluble VD (Wortsman et al. 2000) which may explain an inverse correlation also seen by Vimalaswaran et al (2013) between fat mass and serum 25OHD levels. Long-term regulation of body weight is mainly affected by the degree of obesity (Latner et al. 2000; Montesi et al. 2016). Leptin secretion from fat cells is proportional to the number of stored triglycerides (Park et al. 2016). In the Om group, a significant increase of both TG and leptin levels, at the end of our trial, supports Park et al. (2016) assumption. Later, Vanavanan et al. (2017) correlated between long-term supplementation of n-3FA and the excess ingestion of calories which may cause an increase in fat deposition and prompts an increase in leptin secretion. In this context, overfeeding is known as an inducer of leptin secretion which is stron-

gly associated with insulin resistance (Wang et al. 2001). Insulin resistance in the term of central adiposity is a key link between obesity with T2DM (Gonzalez 2012). This is believed to be essential for the pathogenesis of metabolic syndrome in pre-diabetics, which is characterized by hyperinsulinemia and hypertriglyceridemia (Hirano et al. 2018). Insulin resistance progression could be associated with dysregulated secretion of the anti-diabetogenic activity of leptin (D'Souza et al. 2014). Therefore leptin hypersecretion is a sign of the presence of leptin resistance in the peripheral tissues that develop as obesity progress and early development of IR (Kloting et al. 2014). Insulin insensitivity induced by hyperleptinemia may increase the activity of hormone-sensitive lipase, the enzyme that hydrolyzes stored TG. Concerning the combination of n-3FA and VD₃, a significant elevation of TG levels in the D+Om group was accompanied by an insignificant elevation of leptin levels. We propose that the existence of a synergistic effect for the combination of VD₃ and n-3FA leads to the elevation of serum TG in people with VDD. Elevated 25OHD levels seen in the D+Om group could be involved in dysregulated leptin secretion. However, it is not clear whether central or peripheral action of the supplement was involved in that mentioned association.

Insulin resistance often associates with exhausted pancreatic beta cells due to prolonged insulin hypersecretion because of poor glycemic control in diabetics or prolonged hyperglycemic levels in pre-diabetics (Al-Shoumer et al. 2015). Insulin hypersecretion subsequently down-regulates insulin receptors causing the development of IR in obese subjects (Xu et al. 2007). Likewise, the co-supplementation of VD₃ plus n-3FA perhaps mimics a potential mechanism that stimulates insulin synthesis via VDR in beta cells (Baidal et al. 2016) provoking insulin receptor downregulation. In mice fed with a diet enriched with eicosapentaenoic acid (EPA), higher insulin secretion did not improve glucose tolerance in induced T2DM animal models (Neuman et al. 2017). Unfortunately, due to logistic reasons in this RCT, we did not measure the specific glycemic control parameters including insulin, c-peptide or glycosylated hemoglobin (HbA1c), and these can be considered a trial limitation. Furthermore, one of the limitation that we faced in this study is that, during the trial, there was a significant drop in the participants of control group which was explained previously in the consort diagram. Other

studies were inconsistent either due to dose-dependent (Gray et al. 2013; Poreba et al. 2017), duration of therapy protocol (Jacobo-Cejudo et al. 2017), or the sample allocated for the clinical trial (Hariri et al. 2015). For instance, Poreba et al. (2017) showed that n-3FA therapy with a 520 mg dose for 3 months or with 2gm for 6 weeks did not change leptin levels in T2DM patients. In the systematic review (Farimani et al. 2018) of 10 RCTs involved 494 participants with T2DM treated with doses of (520–740 mg/day n-3FA) an insignificant reduction in leptin levels were observed. However, Farimani et al. (2018) concluded that these RCTs had not reached a definitive conclusion about the effect of n-3FA on the leptin levels in T2DM. Finally, the same dose, 520 mg of DHA + EPA, but for 24 weeks on 54 T2DM patients showed a significant reduction in leptin levels (Jacobo-Cejudo et al. 2017). Remarkably, a significant increase in insulin levels observed by Jacobo-Cejudo et al (2017) has been linked anti-diabetogenic effect of n-3FA supplementation in T2DM patients. However, elevated insulin levels are accompanied by prolonged hyperglycemia (Heianza et al. 2012). Overall, it is unclear whether n-3FA or its combination with VD₃ has anti-diabetogenic effects in people with VDD.

Conclusion

This novel RCT provides that eight weeks of n-3FA therapy significantly declined 25OHD levels which were accompanied by a significant increase in leptin and TG levels. Although the combined effect of VD₃ plus n-3FA, at follow-up, normalized 25OHD levels, notable elevation in TG, and leptin levels were seen. Accordingly, the intervention of VD₃, n-3FA, or their combination may be accompanied by negative effects on leptin resistance. Further clinical trials are required to clarify possible consequences resulted from the co-supplementation of VD₃ and omega on humans' health.

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Supplementary material 1

Supplemental tables

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Data type: Statistical results

Explanation note: S.1. Tukey Test for Post-hoc comparisons of leptin at the end referring to different groups. S.2. Serum PTH, calcium, and PO₄ levels at baseline and follow-up.

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