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A Randomized Controlled Trial of the Effects of Retinyl Palmitate on CD4+ T Cell Related Cytokines, Thyroid Function and Metabolic Biomarkers in Obesity: A Study Design and Protocol

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Abstract

Objective: Obesity, as a low grade chronic inflammatory condition, is associated with immune system dysfunction and several autoimmune diseases. Recent evidence demonstrates a pathogenic role for T-helper cells in developing obesity related immune-disorders. Vitamin A and its retinoid derivates are best known for their immune-regulatory effects. However, the effect of vitamin A supplementation on immune function in obese individuals is still unknown. The aim of the present study is to investigate the possible role of vitamin A supplementation on serum T-helper cytokines and several other secondary outcomes (anthropometric and metabolic parameters and thyroid function) in healthy obese reproductive age women.

Methods: A 16-week interventional trial is conducting on 84 obese (n=56) and non-obese (n=28) women. This trial has two parts: first part, a randomized double blind placebo controlled trial is carrying out on 56 obese women (BMI 30-39.9 kg/m^2). They are randomly allocated to receive either vitamin A (retinyl palmitate 25000 IU/d) or placebo. The second part, comprise a single blind trial conducting on 28 non-obese women (BMI 18.5-24.9 kg/m^2) who are receiving 25000 IU/d retinyl palmitate. The intervention period for both parts is 16 weeks. Serum CD4+ T cell associated cytokines, thyroid hormones and biochemical parameters will be measured in all three groups before and 4 months after intervention.

Results: Several available descriptive findings of the trial are presented here. Serum fasting blood glucose (FBG), triglyceride (TG) and IL-6 concentrations were significantly higher in subjects with higher amounts (≥ 44.27%) of body fat mass (P<0.05).

Conclusion: This is the first trial to investigate the effects of vitamin A supplementation on CD4+ T cells associated cytokines in obese women. The results of the present study will allow for a better clarification of immune-regulatory effects of vitamin A in obesity and might elucidates the potential underlying mechanism.

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Keywords

Vitamin A; Obesity; T-Helpers; Thyroid hormones; Study protocol

Abbreviations

ALT: Alanine Transaminase; AST: Aspartate Transaminase; ATRA: All Trans Retinoic Acid; BMI: Body Mass Index; CBC: Complete Blood Cell Count; CD: Cluster of Differentiation; EDTA: Ethylendiaminetetraacetate; ELISA: Enzyme-Linked Immunosorbent Assay; FBG: Fasting Blood Glucose; HDL: High Density Lipoprotein Cholesterol; HS-CRP: High-Sensitivity C-Reactive Protein; IBD: Inflammatory Bowel Disease; ICL: Immunology Consultants' Laboratory; IFN ©: Interferon γ; IL: Interleukins; IPAQ: International Physical Activity Questionnaire; LDL: Low Density Lipoprotein Cholesterol; NSAID: Non-Steroidal Anti-Inflammatory Drug; PBMCR: Peripheral Blood Mononuclear Cell; RAR: Retinoid Acid Receptors; RBP: Retinol Binding Protein; RXR: Retinoid X Receptors; RCTL: Randomized Controlled Trial; TC: Total Cholesterol; TG: Triglyceride; TGF β: Transforming Growth Factor β; Th: T-Helper; TNF α: Tumor Necrosis Factor α; TTR: Transthyretin; WBC: White Blood Cells; WHR: Waist to Hip Ratio.

Background

Obesity has become a world epidemic over the past 20 years and is growing rapidly worldwide [1]. Obesity has an adverse effect on health; obesity associated co morbidities includes insulin resistance, type 2 diabetes, heart disease, hypertension and dyslipidemia [2,3]. Increasing evidence from several epidemiological studies has linked obesity to proinflammatory conditions and auto immune disease such as inflammatory bowel disease (IBD), psoriasis [4] and rheumatoid arthritis [5].

CD4+ T helper cells, the regulators of immune system, play central role in autoimmune disorders and recent evidence demonstrates a pathogenic role for T-helper cells in developing obesity-related immune disturbances [6]. Recently, it has been observed that the ratio of Th1 and Th2 cells is shifted toward Th1 direction [6-8] and the population of Th17 cells or Th17 related cytokines, IL-17 and IL-23, are increased in obesity [9-11]. Data also support that regulatory T cells may contribute in T helper cells polarization and inhibit the proliferation of Th1 and Th2 cells [12,13].

On the other hand, recent studies have revealed the role of T-helper associated cytokines balance in human reproduction; it has been shown that normal pregnancy is associated with higher serum levels of Th2 associated cytokines, whereas, higher levels of serum Th1 associated cytokines were detected in women with a history of recurrent pregnancy losses [14,15]. Other reports have revealed the role of T-regulatory cells in normal fertility of women; Jasper et al. [16] reported that reduced expression of T-regulatory cell transcription factor Foxp3 in endometrial tissue of women is associated with unexplained infertility.

Considering the above mentioned information, a therapeutic tool

which can normalize the T-helper cells cytokines balance in women of reproductive age would be very useful to overcome the adverse effects of both obesity and immune system dysfunction.

Vitamin A and its derivates (retinoids) modulate the growth and differentiation in mammalian cells via their nuclear hormone receptors mainly known as retinoic acid receptors (RAR) and retinoid X receptors (RXR) [17]. Therapeutic use of vitamin A and its derivates in skin disease, cancer and embryonic development has attracted great attention [18,19]. One of the underlying bases for the importance of vitamin A in health is that vitamin A and other retinoids exert immunomodulatory and anti inflammatory functions; the link between vitamin A and infection has been revealed in population based studies [20,21]. However, the mechanism for these functions in humans is not well understood. Several studies indicate that vitamin A deficiency is associated with impaired immune responses mediated by T-helper type 2 (Th2) cells whereas Th1 mediated responses may be enhanced [22-24]. Because obesity is associated with immune disorders, it is logical to imagine that vitamin A supplementation might modulate the T- helper related immunity in obesity. However, our review of literature was unable to locate a single published randomized controlled trial investigating the therapeutic role of vitamin A on T helper- related cytokines in obesity. The current study will be the first trial of this kind aiming to investigate the effect of daily oral vitamin A supplementation for 16 weeks on serum T helper cell associated cytokines in obese and non obese women. Secondary outcomes include change in anthropometric variables, biochemical parameters and thyroid hormones between vitamin A and placebo groups.

Design and Methodology

Study parts

This study comprises two parts: the first part designed as a randomized double blind placebo controlled trial (RCT) aims to evaluate the effect of vitamin A or placebo on T helper associated cytokines, anthropometric and biochemical variables and thyroid function in obese women; whereas the second part is a randomized single blind trial investigating the effect of vitamin A on above mentioned parameters in non- obese women. The design, methodology and specific aims for two parts are similar.

Participants: First part designed as a randomized double blind placebo controlled trial (RCT) of 56 healthy volunteer obese (BMI 30-39.9 kg/m^2) women aged 20-52 years old, while the second part, as a single blind controlled trial, is conducting on 28 healthy volunteer non-obese women (BMI 18.5-24.9 kg/m^2) in the similar age group. The subjects are invited to participate in the study through invitation letters which are distributed in different parts of Tabriz city, Iran. The rationale of using the dose of 25000 IU and the 16 weeks period of the current study is on previous reports [25] which support that vitamin A supplementation in this dose and duration can increase circulating levels of all trans retinoic acid (ATRA) and 9-cis retinoic acid to concentrations that will interact with their respective receptors (RAR and RXR).

Inclusion criteria: The inclusion criteria are as follows:

1. BMI between 30-39.9 kg/m^2 and WHR>0.8 for obese women.
2. BMI between 18.5-24.9 kg/m^2 and WHR<0.8 for non-obese women.
3. Ages 20-52 years old.

Exclusion criteria: The exclusion criteria are as follows:

1. History of diabetes (FBG>110 mg/dl).
2. Thyroid disorders (TSH >3 or <0.3 m IU/l, T3>3.2 or <1.3 nmol/l and T4>160 or <64 nmol/l).
3. Chronic liver disease (higher than normal concentrations for ALT and AST).
4. Renal disease and autoimmune disease.
5. Hypertension.
6. Any hypersensitivity reaction to vitamin A and its derivates.
7. Women who are pregnant, lactating or are planning for a pregnancy.
8. Following any weight reduction programs.
9. Use of any vitamin A containing dietary supplement or medications (Tretinoin, Isotretinoin, and other all trans retinoic acid derivates) for at least three months prior to participating in the study and during the intervention period.
10. Use of medications within past 3 months that could have an effect on immune system (NSAIDs, immunosuppressive drugs such as corticosteroids, azathioprine, cyclosporine and so on).
11. Use of medications within past 3 months that could have an effect on body weight (β-blockers, antipsychotics, tricyclic antidepressants, cyproheptadine, phenothiazine, benzodiazepines).

Sample size

To our review of literature, there was no preliminary study evaluating the effect of retinyl palmitate supplementation on serum cytokines in obesity; therefore, we calculated the sample size according to a reference paper of all transretinoic acid considering 10 pg/ml differences of IL-4 concentrations between intervention and control group [26]. To detect this difference with 80% power and α-error of 5%, a total of 75 individuals are needed. Allowing for 10% drop-out over 16 weeks of intervention, the total sample size required for the study is 84 individuals.

Randomization

Using random permuted block method (block size 2 or 4), obese individuals are randomly assigned to one of the treatment groups of either vitamin A (retinyl palmitate 25000 IU/d) or placebo. Vitamin A and placebo soft gels are indistinguishable and are packaged in identical bottles each containing 120 capsules. Theses bottles are coding A and B to identify two obese groups. Therefore, participants, investigators and laboratory staff are blind for obese individual's group assignment. Non- obese group are receiving vitamin A and the investigators could not be technically blind to their identify codes. The study duration is 16 weeks. The trial flowchart has been presented in figure 1.

Compliance

All participants are instructed to have their usual diet and physical activity during intervention period. Follow- up procedure is done by monthly phone calls. Compliance will be verified by dividing the actual number of full supplement capsules consumed by subjects in all three groups by the total number of capsules that were provided to be consumed over the 4 months (i.e., 1 capsule /day for 4 months).

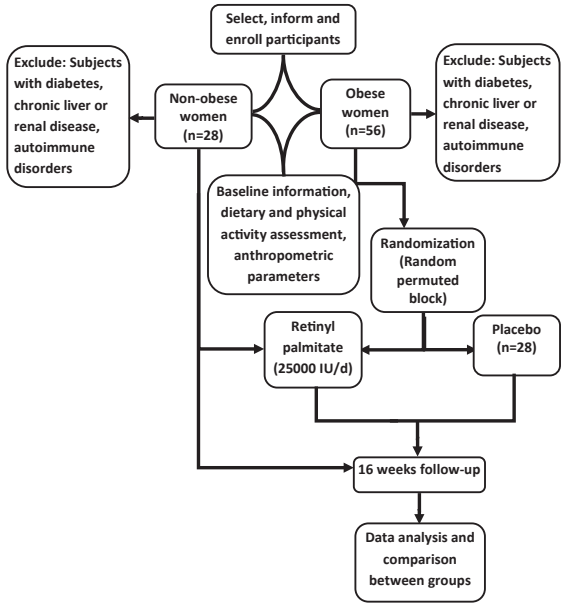

Figure 1: Trial flow chart.

Outcome parameters

Outcome parameters will be measured at baseline and 16 weeks after intervention. All outcome parameters that will be measured are listed below. A summary of the study parameters is also presented in table 1.

Dietary assessment

Dietary intake is assessed by a three-day 24-h dietary recall questionnaire (including a weekend day) at baseline and 16 weeks after intervention.

Physical activity

Physical activity is measured using the short form of international physical activity questionnaire (IPAQ). This face-to-face questionnaire includes the information about three specific types of activity that are walking, moderate-intensity activities and vigorous-intensity activities in a comprehensive set of domains including: leisure time physical activity, domestic and gardening (yard) activities, work-related physical activity and transport-related physical activity. The analysis is based on the total time spent on physical activity in the previous week (metabolic equivalent/minute).

Anthropometric variables

Weight is measured on a calibrated Seca scale (Itin Scale Co., Inc. Germany) with the accuracy of 0.1 kilogram while subjects wearing light clothes and no shoes. Height measurement has taken by a cotton ruler. BMI is calculated as actual weight (kg) divided by height (m^2). The waist measurement is taken above the iliac crest at the natural waistline and the hip measurement is taken at the largest area of the natural hip line. Waist to hip ratio (WHR) is also calculated based on waist and hip measurements.

Body composition analysis

Bio-electrical impedance analysis (BIA) is used to calculate body composition. Measurements are performed at right side of the subjects and after at least 10 minutes resting, while the subjects remove shoes, socks and jewellery and are in supine position. The instrument has 4 electrodes with 1, 5, 10, 50 and 100 KHz signals. Two electrodes are placed at dorsal surface of hands and two other are placed at dorsal surface of foot according to the instructions of manufacturer. In this technique, body fat and fat free compartments include fat mass (FM), fat free mass (FFM), body cell mass (BCM) and extracellular mass (ECM). Water compartments also include total body water (TBW), intracellular water (ICW) and extracellular water (ECW). Measurements are performed by Human-IM Plus model N-0166 (DS Dietosystem Medi Group, Milan, Italy). Subjects with pacemaker will be excluded from analysis.

Table 1: A summary of study variables.

Parameter	Components	Methods / sample type
Demographic and lifestyle information	-Birth date and location	Questionnaire
	-Contact information	
	-Tobacco use and smoking	
	-Marital status	
	-Pregnancy and lactation	
	-History of medication use	
	-Informed consent	
	-professional sports	
Nutritional status	-Dietary intake in meals and snacks	3 day 24-h recall questionnaire
Anthropometric data	-Weight, height, waist and hip circumference	Calibrated Seca scale and cotton ruler
Physical activity score	-Vigorous, intermediate and mild physical activities	International physical activity questionnaire
Clinical data	-Cytokines	Serum
	-Hematologic parameters	Whole blood
	-Lipid profile	Serum
	-Thyroid hormones	Serum
	-CRP	Serum
	-Angiotensin II	Serum
	-AST and ALT	Serum
	-RBP and TTR	Serum
Compliance	Information about the regular use of supplement	Number of full supplement capsules consumed divided by the total number of capsules

CRP: C-reactive Protein; AST: Aspartate Transaminase; ALT: Alanine Transaminase; RBP: Retinol Binding Protein; TTR: Transthyretin

Biochemistry measurements

After an overnight fasting (10-12 hours) at 8:30-9:30 a.m. in the morning venous blood samples (20.0 ml) are collected. Three (3.0) ml of blood samples is immediately transferred to ethylendiaminetetraacetate (EDTA) specimen bottles for hematological parameters measurement. The remaining blood is put into a dry tube for serum extraction and analysis of biochemical profile. Sera are transferred into clean microtubes in aliquots. All parameters that will be measured are listed below:

Complete blood cell count (CBC) assay: CBC test including measurements of total and differential white blood cell count (WBC), hemoglobin, hematocrit, platelet count and red blood cell count which are determined using an automatic cell counter (Drew Scientific, Excell-18; Minnesota, USA).

Cytokine assay: Serum concentrations of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17A, tumor necrosis factor α (TNF α), interferon γ (IFN γ), and transforming growth factor β (TGF β) are determined using enzyme-linked immunosorbent assay kits (Quantikine immunoassay; e-Bioscience, San Diego, CA).

Retinol binding protein (RBP) and Transthyretin (TTR): RBP and TTR are also determined by ELISA kit (Immunology consultants' laboratory (ICL), Newberg, USA). The RBP4 to TTR ratio is calculated by dividing the plasma RBP4 concentration by the plasma TTR concentration. For calculation of the molar concentrations of RBP 4 and TTR a molecular mass of 21000 g/mol for RBP 4 and 54000 g/mol for TTR are used [27,28].

Fasting blood glucose (FBG) and Lipid profile: FBG and serum lipids including total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL) are measured by enzymatic methods (Pars-Azmoon; Tehran, Iran).

Aspartate transaminase (AST) and Alanine transaminase (ALT): Serum AST and ALT levels are also determined by enzymatic methods (Kits from Pars-Azmoon, Tehran, Iran).

C-reactive protein (CRP): High-sensitivity C-reactive protein (HS-CRP) is measured by ELISA method (DRG Instruments GmbH, Germany).

Angiotensin II: Serum angiotensin II level is measured by ELISA method (ENZO, Life Science Inc).

Statistical analysis

All analysis will be performed using SPSS software (Version 17, SPSS Inc., Chicago, IL, USA). Normality of data will be tested with the Kolmogorov-Smirnov test. A two-sided P value less than 0.05 will be considered statistically significant. Comparison of variables between groups will performed by means of one way ANOVA for normal distribution and Kruskal-Wallis test otherwise. Paired sample t-test will also be used to determine the effect of vitamin A on biochemical parameters.

Ethical considerations

The participants are informed about the purpose and possible hazards of the trial and are free to leave the study at any time. Written informed consent of Tehran University of Medical sciences is obtained from all of the participants before enrolling. The study is approved by ethics committee of Tehran University of Medical Sciences (Project number 89-04-27-11869). This trial has also been registered at Clinicaltrials.gov (Identifier NCT-01405352).

Descriptive findings

Several available descriptive results of the study are presented in tables 2, 3 and 4. Subjects were categorized based on their body fat mass percent into four quartiles. Comparison of the baseline characteristics of anthropometric and biochemical variables between subjects were based on the subjects fat quartiles. Table 1 presents the demographic and anthropometric characteristics of study participants. Age was not significantly different between groups; however, other anthropometric variables were significantly different ($P<0.05$). Serum FBG, TG and IL-6 concentrations were significantly higher in group with the highest body fat mass compared with other groups (Table 3). However serum IL-10 and IFN-γ in group II and III were in their highest concentrations compared with other groups (Table 4).

Discussion

Obesity is associated with adverse health conditions including type 2 diabetes, dyslipidemia, cardiovascular disease and poor health in general [29]. Moreover, obesity is associated with alterations in immune responses and a stage of chronic low grade inflammation and T-helper cell imbalance as the key regulators of immune response which seems to be partly responsible for many of the above mentioned pathophysiologic conditions [30]. In the present study, we reported our available descriptive results and to our findings serum IL-6 and IFN-γ concentrations were higher in subjects with higher body fat mass. However, the results of researches about the effects of obesity on immune system are conflict probably because of their focusing on measurement of several classic markers of inflammation such as CRP, IL-6 and TNF-α in obese individuals in a cross-sectional fashion [30,31]. However, these aspects provide limited information about the markers of immune function in obesity [30]. Evaluation

Table 2: Descriptive characteristics of trial participants categorized based on body fat mass quartiles.

	Group (Percent of body fat mass %)				*P†*
	I (≤ 31.80%)	II (31.80-39.25%)	III (39.25-44.27%)	IV (44.27% ≤)	
n	22	20	21	21	
Age (years)	35.50 ± 6.74	34.5 ± 8.90	38.85 ± 6.0	35.47 ± 6.7	NS
Weight (kg)	58.01 ± 9.2	70.43 ± 7.3	78.31 ± 7.7	87.43 ± 8.3	<0.001
BMI (kg/m²)	22.71 ± 3.6	27.79 ± 2.5	31.22 ± 2.3	35.91 ± 3.4	<0.001
WC (cm)	75.31 ± 8.1	85.87 ± 6.1	93.14 ± 5.8	99.66 ± 6.4	<0.001
HC (cm)	98.22 ± 7.7	106.20 ± 5.14	111.76 ± 5.96	119.80 ± 8.56	<0.001
WHR	0.76 ± 0.03	0.80 ± 0.03	0.83 ± 0.04	0.83 ± 0.02	<0.001

BMI: Body Mass Index; WC: Waist Circumference; HC: Hip Circumference; WHR: Waist to Hip Ratio;
†Compared by One Way ANOVA

Table 3: Comparison of baseline concentrations of metabolic parameters categorized based on body fat mass quartiles.

	Group (Percent of body fat mass %)				*P†*
	I (≤ 31.80%)	П (31.80-39.25%)	Ш (39.25-44.27%)	IV (44.27% ≤)	
n	22	20	21	21	
FBG (mmol/L)*	4.09 ± 0.50	4.41 ± 0.65	4.24 ± 0.71	4.79 ± 1.19	0.035
TC (mmol/L)	3.77 ± 0.76	3.93 ± 0.71	4.16 ± 0.86	4.47 ± 1.29	NS
TG (mmol/L) *	1.18 ± 0.43	1.43 ± 0.57	1.36 ± 0.47	1.85 ± 0.67	0.001
LDL-C (mmol/L)	1.92 ± 0.59	1.84 ± 0.62	2.10 ± 0.74	2.19 ± 0.03	NS
HDL-C (mmol/L)	1.29 ± 0.22	1.43 ± 0.31	1.43 ± 0.18	1.42 ± 0.21	NS
AST (U/l)	19.90 ± 8.91	19.95 ± 6.98	22.57 ± 7.22	19.57 ± 3.85	NS
ALT (U/l)	15.81 ± 8.35	16.60 ± 6.96	18.09 ± 8.17	17.80 ± 7.48	NS

FBG: Fasting Blood Glucose; LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; ALT: Alanine Transaminase; AST: Aspartate Transaminase; IL-6: Interleukin 6; † Compared by One Way ANOVA; *Group IV has significantly higher levels than Group I (Multiple comparisons with Bonferroni Corrections).

Table 4: Comparison of baseline concentrations of pro-inflammatory and anti-inflammatory cytokines categorized based on body fat mass quartiles.

	Group (Percent of body fat mass %)				*P†*
	I (≤ 31.80%)	П (31.80-39.25%)	Ш (39.25-44.27%)	IV (44.27% ≤)	
N	22	20	21	21	
IL-1β (pg/ml)	3.59 ± 1.77	3.45 ± 1.59	2.96 ± 1.18	3.67 ± 1.61	NS
IL-2 (pg/ml)	13.01 ± 5.77	14.70 ± 6.45	9.95 ± 4.19	13.00 ± 9.93	NS
IL-4 (pg/ml)	2.48 ± 0.67	2.74 ± 0.99	2.22 ± 0.65	2.14 ± 0.5	NS
IL-6 (pg/ml) *	29.46 ± 8.10	28.39 ± 5.60	27.12 ± 5.16	32.69 ± 4.52	0.023
IL-10 (pg/ml) **	5.13 ± 2.1	8.8 ± 2.9	4.3 ± 1.25	3.9 ± 1.21	0.037
IL-17 (pg/ml)	14.36 ± 5.9	13.23 ± 7.2	12.63 ± 4.90	13.31 ± 6.3	NS
TGF-β (ng/ml)	2.98 ± 0.86	3.20 ± 0.98	3.01 ± 0.91	3.30 ± 1.01	NS
IFN-γ (pg/ml)***	90.12 ± 15.92	75.23 ± 15.23	174.79 ± 47.43	87.75±13.09	0.05
TNF-α (pg/ml)	0.25 ± 0.03	0.30 ± 0.08	0.25 ± 0.19	0.21 ± 0.01	NS

IL: Interleukin; TGF β: Transforming Growth Factor β; IFN γ: Interferon γ; TNF-α: Tumor Necrosis Factor α †Compared by Kruskal-Wallis Test; * Group IV has significantly higher levels than other Groups; ** Group П has significantly higher levels than other Groups; ***Group IV has significantly higher levels than other Groups (Multiple comparisons with Bonferroni Corrections)

of the immune function especially the $CD4^+$ T-cell function in an interventional design is the best method for assessment the usefulness of a therapeutic agent in improving the immunity in obese individuals. Vitamin A and its synthetic derivates have been shown to modulate immunity through developing human Th2 and T-regulatory cytokine response and suppressing the Th1 and Th17 cytokine production in several *in vitro* [26,32,33] or *in vivo* [34,35] studies. For example, in the study by Dawson et al. [26] treatment of human PBMCs with all transretinoic acid increased the mRNA and protein levels of IL-4, IL-13 and decreased the levels of IFN-γ and IL-2 levels. In another study by Aukrust et al. [34] supplementation with a 6500 IU/d dose of vitamin A for 6 months increased IL-10 and decreased TNF-α levels in patients with common variable immunodeficiency (CVI). However, our study will be the first clinical trial investigating the effect of vitamin A supplementation on $CD4^+$ T cell cytokines in obesity. Moreover, our study will investigate the effects of vitamin A supplementation on the extensive anthropometric, biochemical and metabolic markers of obesity including weight, body composition, thyroid hormones, lipid profile, liver enzymes, CRP and immune system cytokines.

The strengths and limitations of the present study include: randomized double blind placebo controlled design of the study provides protection against various confounders; furthermore, all analysis will be adjusted for potential confounding factors include physical activity, dietary intake of nutrients and antioxidants and difference in baseline concentrations of biochemical parameters between groups. On the other hand, the results of the present trial will only be relevant for reproductive age women, the results may not be directly applicable for men or even for women in other age groups; also, the sensitivity of cytokine assay in serum at the detectable ranges of kits might make it difficult to detect biologically significant differences of cytokines between treatment groups.

Conclusions

In this trial, we will assess the effect of four months treatment with 25000 IU retinyl palmitate on the immune system in obesity. We believe that the results of the present study will provide useful information about the immune regulatory effects of vitamin A in obesity and might confirms the therapeutic role of vitamin A in obesity induced-immune disorders specially auto immune disease.

Trial Status

The trial was first designed and started at 2011. The study is ongoing. The subject recruitment has been completed; however, biochemical assays and data analysis is still incomplete.

Competing Interests

The authors declare no competing interest.

Authors' Contributions

Ali Akbar Saboor-Yaraghi and Mahdieh Abbasalizad Farhangi have main roles in developing the general idea of the project. Ali Akbar Saboor-Yaraghi and Seyyed Ali Keshavarz have supervised and directed the project. Mahdieh Abbasalizad Farhangi has carried out the project and written the first draft of the manuscript, Mohammadreza Eshraghian carried out statistical analysis and Alireza Ostadrahimi was involved in patient recruitment. All authors consented to the publication of the manuscript.

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