Biological Trace Element Research (2021) 199:2590–2601
https://doi.org/10.1007/s12011-020-02400-8

Changes in Bone Turnover, Inflammatory, Oxidative Stress, and Metabolic Markers in Women Consuming Iron plus Vitamin D Supplements: a Randomized Clinical Trial

Behnaz Abiri[1] · Mohammadreza Vafa[2] · Fatemeh Azizi-Soleiman[3] · Morteza Safavi[3] · Seyyed Morteza Kazemi[4] · Masood Salehi[5] · Farid Zaeri[6] · Homa Sadeghi[7]

Received: 31 July 2020 / Accepted: 16 September 2020 / Published online: 25 September 2020

Abstract

We aimed to investigate whether combination of vitamin D and iron supplementation, comparing vitamin D alone, could modify bone turnover, inflammatory, oxidative stress, and metabolic markers. Eighty-seven women with hemoglobin (Hb) ≤ 12.7 g/dL and 25OHD ≤ 29 ng/mL vitamin D deficiency/insufficiency aged 18–45 years were randomly assigned into two groups: (1) receiving either 1000 IU/day vitamin D3 plus 27 mg/day iron (D-Fe); (2) vitamin D3 plus placebo supplements (D-P), for 12 weeks. In D-Fe group, significant decrease in red blood cells (RBC) ($P = 0.001$) and hematocrit (Hct) ($P = 0.004$) and increases in mean corpuscular hemoglobin concentration (MCHC) ($P = 0.001$), 25OHD ($P < 0.001$), osteocalcin ($P < 0.001$), high-density cholesterol (HDL) ($P = 0.041$), and fasting blood sugar (FBS) ($P < 0.001$) were observed. D-P group showed significant decrease in RBC ($P < 0.001$), Hb ($P < 0.001$), Hct ($P < 0.001$), mean corpuscular volume (MCV) ($P = 0.004$), mean corpuscular hemoglobin (MCH) ($P < 0.001$), MCHC ($P = 0.005$), serum ferritin ($P < 0.001$), and low-density cholesterol (LDL) ($P = 0.016$) and increases of 25OHD ($P < 0.001$), osteocalcin ($P < 0.001$), C-terminal telopeptide (CTX) ($P = 0.025$), triglyceride (TG) ($P = 0.004$), FBS ($P < 0.001$), and interleukin-6 (IL-6) ($P = 0.001$) at week 12. After the intervention, the D-P group had between-group increases in mean change in the osteocalcin ($P = 0.007$) and IL-6 ($P = 0.033$), and decreases in the RBC ($P < 0.001$), Hb ($P < 0.001$), Hct ($P < 0.001$), and MCV ($P = 0.001$), compared with the D-Fe group. There were significant between-group changes in MCH ($P < 0.001$), MCHC ($P < 0.001$), ferritin ($P < 0.001$), and serum iron ($P = 0.018$). Iron–vitamin D co-supplementation does not yield added benefits for improvement of bone turnover, inflammatory, oxidative stress, and metabolic markers, whereas, vitamin D alone may have some detrimental effects on inflammatory and metabolic markers. IRCT registration number: IRCT201409082365N9

Keywords Vitamin D · Iron · Bone turnover · Inflammation · Oxidative stress

✉ Mohammadreza Vafa
vafa.m@iums.ac.ir

Behnaz Abiri
behnaz.abiri@yahoo.com

Fatemeh Azizi-Soleiman
fatemehazizi87@gmail.com

Morteza Safavi
dr.mortezasafavi@aol.com

Seyyed Morteza Kazemi
Morteza@sbmu.ac.ir

Masood Salehi
salehi.m@iums.ac.ir

Farid Zaeri
fzayeri@yahoo.com

Homa Sadeghi
homa.sadeghi@gmail.com

[1] Department of Nutrition, Faculty of Paramedicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[2] Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Shahid Hemmat Highway, Tehran P.O.BOX: 1449614535, Iran

[3] Department of Clinical Nutrition, School of Nutrition & Food Science, Isfahan University of Medical Sciences, Isfahan, Iran

[4] Bone, Joint, and Related Tissue Research Center, Akhtar Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[5] Department of Biostatistics, School of Public Health, Iran University of Medical Sciences, Tehran, Iran

[6] Department of Biostatistics, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[7] School of Public Health, Department of Nutrition and Food Science, Texas Woman's University, Denton Campus, Denton, TX 76204, USA

Introduction

Vitamin D deficiency and anemia are problems that are still common in the world. It has been estimated that more than a billion people around the world suffer from hypovitaminosis D (25OHD ≤ 75 nmol/L (< 30 of ng/mL))[1].Furthermore, the global prevalence of anemia was estimated at 1.62 billion people between1993 and 2005, mostly caused by iron deficiency [2].

Vitamin D has several valuable functions. Severe vitamin D deficiency (25OHD ≤ 15 nmol/mL) causes rickets in children and osteomalacia in adults, and eventually bone fractures [3]. The reason is attributed to secondary hyperparathyroidism and a consequent increase in bone turnover. Vitamin D supplementation may improve bone mass density and reduce bone turnover and risk of fracture [4]. Additionally, the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-2, interleukin-9, and interleukin-22, is inhibited by vitamin D, whereas the production of anti-inflammatory cytokines such as interleukin-3, interleukin-4, interleukin-5, and interleukin-10 is initiated by vitamin D [5]. It has also been demonstrated that risk of diseases associated with increased DNA damage, caused by oxidative stress, is more in patients with vitamin D deficiency or insufficiency [6]. Researchers suggested that vitamin D may improve some of cardiovascular disease risk factors such as serum lipids, fasting plasma glucose, and oxidative stress [7, 8].

Recent years, the relationship between anemia and vitamin D deficiency has attracted a great deal of attention from researchers. A low level of vitamin D in Asian children with IDA has been reported [9, 10]. A recent systematic review showed a positive relationship between iron status and vitamin D [11]. One of the possible explanations that has been discussed in the literature is the role of iron in the hydroxylation of vitamin D by cytochrome P450 isoforms [12]. These enzymes are dependent on electron donors including ferredoxin and ferredoxin reductase which contain heme group. The effect of iron on vitamin D activation is controversial; intramuscular injection of iron in iron-deficient infants increased vitamin D levels after 2 months[13], while treatment of iron deficiency in adult women had no effect on 25(OH)-D concentrations [14]. Additionally, iron as a cofactor for lysyl prolyl hydroxylase enhances bone strength [15] and its chronic deficiency may lead to osteoporosis in later life. A positive relationship between bone mass density at the proximal radius and serum ferritin levels has been reported in adolescent girls [16].

To the best of our knowledge, few interventional studies have investigated the effects of iron supplementation on vitamin D concentration, bone turnover, metabolic, inflammatory, and oxidative stress biomarkers. The present study aimed to investigate whether combination of vitamin D and iron supplementation, comparing vitamin D and placebo, could modify vitamin D status, some bone turnover markers, selected markers of inflammation and oxidative stress, lipid profiles, and glucose level in child-bearing age women with low levels of hemoglobin (Hb) and vitamin D.

Materials and Methods

This study was a triple-blind, randomized, clinical trial conducted in Kermanshah, Iran, during May–August 2015. Seven hundred women aged 18–45were recruited at health care centers and screened for vitamin D deficiency and anemia. Inclusion criteria were as follows: healthy women, non-smoker, non-pregnant, non-lactating, have a body mass index (BMI) between 18.5 and 29.9 kg/m^2, with Hb ≤ 12.7 g/dL and 25(OH)-D < 30 ng/mL. Iron deficiency anemia is diagnosed with hemoglobin levels less than 12 g/dL. However, Beutler and Waalen have suggested considering the altitude of the residence site of people for the screening of anemia [17]; as this cut-off could be increased to 4% per 1000 meters above sea level [18], Hb ≤ 12.7 g/dL was used for anemia screening. Vitamin D deficiency was defined as 25OHD < 20 ng/mL and vitamin D insufficiency as a level of 20–29 ng/mL [19].

Females were excluded if they had amenorrhea, menopause, thalassemia, hemochromatosis, inflammatory bowel diseases, Crohn's disease, gastric ulcers, celiac disease, gastrointestinal bleeding disorders, kidney diseases, blood donation, or taking any kind of the medications with an impact on hematologic and biochemical markers during the 4 past months (i.e., dietary supplements and drugs like nonsteroidal anti-inflammatory, anti-diabetic, or lipid-lowering agents). The sample size was calculated for detecting a 21.1 ng/mL increase [20] in the concentrations of vitamin D in Fe-D group as compared with P-D group, according to the dose of vitamin D used in the present study, at a significance level of 0.05 and 90% power. A study size of 76 females was obtained. Assuming a 25% drop-out rate, a total of 100 women participated in the intervention. As most of screened women had normal hemoglobin levels (≥ 12.7 g/dL), totally, 100 women were recruited onto the trial. Baseline characteristics of participants are shown in Table 1.

This study was in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of the Isfahan University of Medical Sciences (IR.mui.rec.1394.3/291) and the Iran University of Medical Sciences (IR.IUMS.REC.1394.25971). Written informed consent was obtained from all participants after explaining the purpose and process of the study, and the intervention was registered at www.irct.ir, IRCT201409082365N9. This study followed the CONSORT guidelines and the details of trial are presented in Fig. 1.

Table 1 Characteristics of the participants at baseline according to randomly assigned group

	All (n = 87)	D-Fe (n = 48)	D-P (n = 39)	P value
Age, year	34. 6 ± 7.4	34.1 ± 7.6	35.2 ± 6.7	0.094*
Married, n (%)	62 (62.0)	30 (62.0)	32 (64.0)	0.267*
Body weight, kg	70.2 ± 14.5	70.7 ± 14.9	69.7 ± 14.1	0.714*
BMI, kg/m^2	27.2 ± 1.2	26.6 ± 1.2	27.8 ± 1.2	0.304*
Physical activity level, MET/h	1459.1 ± 2946.6	1430.7 ± 2062.2	1484.9 ± 3591.0	0.892*

Data are expressed as mean ± SD except for marriage status

BMI body mass index, *MET* metabolic equivalent

*Obtained from independent samples *T* test

An independent researcher used a random allocation sequence to randomly allocate females in a 1:1 ratio to receive 1000 IU cholecalciferol and 27 mg ferrous sulfate or 1000 IU cholecalciferol and matched placebo in terms of shape, color, and taste for 12 weeks. Vitamin D, iron, and placebo tablets were manufactured by Jalinous (Jalinous pharmaceutical Company, Tehran, Iran), Maad (Maad Pharmacy, Tehran, Iran), and Tehran University of Medical Sciences (Roshd Pharmaceutical Incubation Center, Tehran, Iran), respectively. The independent researcher packaged iron and placebo tablets in boxes and consecutively numbered according to a computer-generated randomization list. Using opaque envelopes, the boxes were delivered to the study supervisor for random allocation. An order number was assigned to each female and the supplements were given in the corresponding pre-packed box every 4 weeks. The allocation was masked from the participants, study supervisor, staff involved in outcome assessment, and statistician until drafting results.

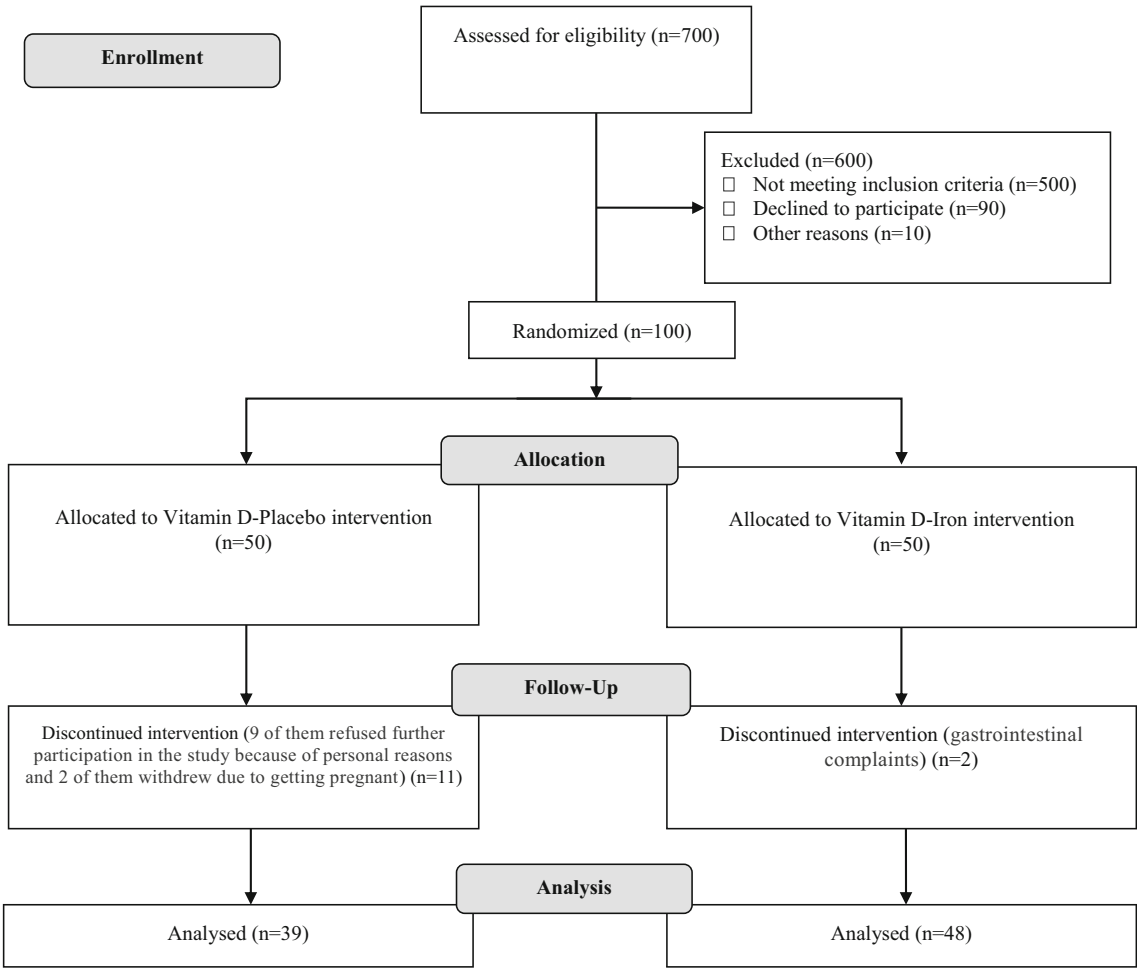

Fig. 1 Flowchart of participants

Women were instructed to take both tablets daily and not change their habitual dietary intake and physical activity. Compliance with the intervention was assessed using the levels of 25OHD, serum iron, and number of remaining pills. Additionally, all participants were contacted monthly during the study period.

Primary Outcome

The concentration of 25OHD was measured at baseline and 12 weeks. Blood samples were collected at the time of screening and at the end of the intervention after a 12-h fasting period. Whole blood (3 cc) was used to assess complete cell blood count. Serum was obtained from remaining blood after centrifugation at 1000*g* for 15 min, stored at – 80 °C, and analyzed after the study was completed. Serum vitamin D was measured by an ELISA kit, with intra- and inter-assay coefficients of variation of 5.6 and 6.4%, respectively (25-hydroxyvitamin D EIA, Immunodiagnostic Systems, IDS, UK).

Secondary Outcome

Secondary outcomes included hematologic indices (complete cell blood count, ferritin, serum iron, transferrin saturation, and total iron binding capacity (TIBC)); bone turnover markers (osteocalcin, C-terminal telopeptide (CTX), parathyroid hormone (PTH), alkaline phosphatase (ALP) enzyme activity and enzyme mass); inflammatory markers (interleukin-6, TNF-α, high-sensitivity C-reactive protein (hsCRP)); oxidative stress markers (total antioxidant capacity (TAC) and malondialdehyde, fasting blood sugar (FBS)); and lipid profile (total cholesterol (TC), low-density cholesterol (LDL), low-density cholesterol (HDL), and triglyceride (TG)). Measurement of ferritin was done by an ELISA kit. Sensitivity and intra-assay coefficient of variation for ferritin were 0.5 ng/mL and 8.7%, respectively. Serum iron was measured using enzymatic photometry. Transferrin was defined by the immunotorbidimetric method.

The bone formation marker, serum osteocalcin, was measured by an ELISA commercial kit with 0.25 ng/mL sensitivity and intra-assay coefficient of variation of 5.7% (ZellBio GmbH, Ulm, Germany). The bone resorption marker, serum CTX, was determined by an ELISA commercial kit with 10 pg/mL sensitivity and intra-assay coefficient of variation of 6.1% (ZellBio GmbH, Ulm, Germany). PTH was measured with the use of an ELISA kit (ZellBio GmbH, Ulm, Germany, sensitivity: 0.8 pg/mL and intra-assay coefficient of variation: 5.4%). ALP mass was defined using an ELISA commercial kit (ZellBio GmbH, Ulm, Germany, sensitivity: 0.1 ng/mL and intra-assay coefficient of variation: 5.8%), and ALP activity was measured by colorimetry.

hsCRP was measured with the use of an ELISA kit (Diagnostics Biochem Canada Inc., Ontario, Canada, sensitivity: 10 ng/mL and intra-assay coefficient of variation: 6.2%). Serum interleukin-6 was determined by an ELISA commercial kit with 2 pg/mL sensitivity (Diaclone, Besancon, France). Serum TNF-α was measured by an ELISA commercial kit with 8.5 pg/mL sensitivity and intra-assay coefficient of variation of 6.6% (Diaclone, Besancon, France). TAC was defined as using an ELISA commercial kit (ZellBio GmbH, Ulm, Germany, sensitivity: 0.1 mM and intra-assay coefficient of variation: 5.1%), and malondialdehyde was measured by an ELISA commercial kit (ZellBio GmbH, Ulm, Germany, sensitivity: 0.1 mM and intra-assay coefficient of variation: 4.8%). FBS and lipid profiles were measured by enzymatic colorimetric methods.

Height was measured to the nearest 0.1 cm using a stadiometer. Body weight was measured to the nearest 100 g on a scale. BMI was calculated as weight (kg)/height $(m)^2$. The body fat mass was determined with the same scale used for measuring weight. At the beginning and at the end of the intervention, dietary intakes were assessed with a 24-h food recall for 3 days (2 weekdays and 1 weekend day) and energy, macronutrients, and micronutrient intakes were estimated by nutritionist 4 software. Physical activity level was assessed using International Physical Activity Questionnaire, short format [21], at baseline and the end of the study. Sun exposure was defined by a validated questionnaire [22].

Statistical Analysis

Statistical analyses were performed using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Cor), and P value < 0.05 was considered statistically significant. Normal distribution of the outcome variables was defined using the Shapiro–Wilk test. Changes within the groups from baseline to week 12 were analyzed using paired samples T tests or Wilcoxon signed rank test whenever the distributions of the variables were not normal. Change on each variable was calculated as pre-intervention minus post-intervention values for each subject, and then mean value for the change for each group was calculated. To compare mean changes in variables between groups, the Mann–Whitney or independent samples T test was used. One-way analysis of covariance (ANCOVA) was also conducted to adjust the effect of baseline differences on the intervention results, whenever there were significant differences in baseline values between groups. Continuous variables are presented as mean and standard deviations (mean ± SD). Categorical variables are demonstrated as frequencies and percentages. Chi-square test was used to assess the differences between categorical variable.

Results

Ages of the volunteers were 35.18 ± 6.74 and 34.08 ± 7.64 years for the D-P and D-Fe groups, respectively, without

significant differences between the groups (Table 1). No significant differences between groups were found in body weight, BMI, and physical activity level at baseline.

A total of 87 women completed the study. Eleven women dropped out in the D-P group; nine of them refused further participation in the study because of personal reasons and two of them withdrew due to getting pregnant. Two females in the D-Fe group were excluded due to gastrointestinal complaints.

Table 2 shows the intervention results. There were significant differences in baseline values of red blood cells (P = 0.013), MCV (P = 0.006), MCH (P = 0.006), ferritin (0.041), alkaline phosphatase enzyme mass (P = 0.034), alkaline phosphatase enzyme activity (P = 0.033), CTX (P = 0.032), parathyroid hormone (P = 0.045), hsCRP (0.007), total antioxidant capacity (P = 0.005), total cholesterol (P = 0.008), triglyceride (P = 0.011), LDL (P = 0.006), and HDL (P = 0.008) between D-Fe and D-P groups.

In the D-Fe group, significant decreases in red blood cells (P = 0.001), hematocrit (P = 0.004), and weight (P = 0.012) and increases in 25OHD (P < 0.001), MCHC (P = 0.001), osteocalcin (P < 0.001), fasting blood sugar (P < 0.001), and HDL (P = 0.041) were observed. The D-P group showed significant decrease of RBC (P < 0.001), Hb (P < 0.001), hematocrit (P < 0.001), MCV (P = 0.004), MCH (P < 0.001), MCHC (P = 0.005), serum ferritin (P < 0.001), and LDL (P = 0.016) and increases of 25OHD (P < 0.001), osteocalcin (P < 0.001), CTX (P = 0.025), fasting blood sugar (P < 0.001), interleukin-6 (P = 0.001), and triglyceride (P = 0.004) at week 12.

After the intervention, the D-P group had between-group decreases in mean change in the red blood cells (P < 0.001), hemoglobin (P < 0.001), hematocrit (P < 0.001), and MCV (P = 0.001), and increases in the osteocalcin (P = 0.007) and interleukin-6 (P = 0.033), compared with the D-Fe group. There were significant between-group changes in MCH (P < 0.001), MCHC (P < 0.001), ferritin (P < 0.001), and serum iron (P = 0.018). Though MCH, MCHC, and serum iron increased in the D-Fe group, these variables decreased in the D-P group. Controlling for pre-intervention variance by ANCOVA did not change the results for indices that had different initial values, except for triglyceride. The increase in triglyceride concentration was higher in the D-P group after adjusting for baseline values (P = 0.016).

Dietary characteristics of the females at baseline and week 12 are presented in Table 2. There were no changes in the intake of macro- and micronutrients at week 12 compared with baseline (Table 3). Sun exposure was similar in all volunteers at baseline and after 12 weeks of intervention (Table 4).

Discussion

This study examined the advantages of adding iron to vitamin D vs. vitamin D alone on bone turnover, inflammatory, oxidative stress, and metabolic markers. The intervention was performed in women with low levels of hemoglobin and 25(OH)-D. However, iron plus vitamin D was not superior to vitamin D alone.

The usefulness of iron therapy in the treatment of anemia has been proven, especially in those with deteriorated hematologic condition [23]. However, supplementation with high dose of ferrous sulfate in female athletes for 11 weeks did not result in an improvement in hematologic parameters and had only a preventive effect on reducing body iron which is in line with the present study [24]. In our study, minor decreases in hematologic indices were observed in the D-Fe group that is physiologically implausible. It has also shown that serum levels of transferrin saturation, ferritin, and hemoglobin diminished by using a fortified food product containing calcium and vitamin D during 9 weeks [25]. Failure to see an increase in these variables in the present study could be related to menstruation [26] and insufficient iron dose used [27]. Further studies with different groups and interventions are required.

The salutary effect of vitamin D on bone health is well known. Vitamin D controls bone remodeling through inducing ligand receptor activator of NF-kB, regulation of phosphate homeostasis by increasing fibroblast growth factor 23, and increases bone response to mechanical stimulation via mitogen-activated protein kinase signaling pathway [28]. It has shown that consumption of 7000 IU/day cholecalciferol for 26 weeks in vitamin D–deficient women can reduce PTH and CTX and increase arm bone mass density [29]. A recent study indicated that 1000 IU/day vitamin D supplementation for 9 months decreased bone turnover markers [30]. Lerchbaum et al. evaluated the effects of 20,000 IU/week vitamin D on bone turnover markers in 200 healthy men with 25OHD < 75 nmol/L in comparison to placebo. However, after 12 weeks of supplementation, there was no significant effect on bone metabolism or density [31]. In our study, it was assumed that vitamin D plus iron supplementation versus vitamin D alone would further increase 25OHD concentration, further improve bone formation, and further reduce bone resorption. We observed that both groups experienced approximately the same increase of 25OHD, bone turnover markers rose in both groups, and surprisingly, the bone formation marker, osteocalcin, was significantly higher in the D-P group. There are limited studies evaluating the effects of iron on 25OHD and bone turnover markers. Published results of the Safe-D study indicated that factors like age, body composition, and iron status have impact on bone turnover markers [32]. Blanco-Rojo et al. reported no effects of iron-fortified juice consumption on bone turnover markers in women with ferritin < 40 ng/mL [33]. The 25OHD levels reduced compared with baseline in both groups, with no difference between groups. In rats fed a diet low in iron, a sharp reduction in the concentration of procollagen type I N-terminal

Table 2 The effects of vitamin D–placebo vs. vitamin D–iron co-supplementation on the hematologic, bone turnover, inflammatory, oxidative stress, and metabolic biomarkers

Variable	D-placebo (n = 39)				D-Fe (n = 48)				P value*
	Baseline	Week 12	P value†	Mean change	Baseline	Week 12	P value†	Mean change	
RBC, × 1012/L	5.1 ± 0.6	4.7 ± 0.5	< 0.001	− 0.4 ± 0.3	4.8 ± 0. 6	4.6 ± 0.5	0.001	− 0.2 ± 0.3	0.005‡
Hb, g/dL	12.1 ± 1.1	10.6 ± 1.0	< 0.001	− 1.5 ± 0.9	12.3 ± 1.0	12.0 ± 1.2	0.204	− 0.2 ± 1.2	< 0.001
Hct, %	38.4 ± 2.9	34.5 ± 2.9	< 0.001	− 3.9 ± 2.8	38.4 ± 2.6	37.1 ± 2.7	0.004	− 1.3 ± 3.0	< 0.001
MCV, fL	76.4 ± 8.8	74.7 ± 10.2	0.004	− 1.7 ± 3.5	81.4 ± 7.8	81.2 ± 7. 6	0.782	− 0.1 ± 3.3	0.038‡
MCH, pg	24.0 ± 3.3	22.9 ± 3.5	< 0.001	− 1.1 ± 1.2	25.9 ± 3.0	26.3 ± 2.9	0.102	0.4 ± 1.5	< 0.001‡
MCHC, g/dL	31.4 ± 1.0	30.7 ± 1.4	0.005	− 0.6 ± 1.3	31.8 ± 1.2	32.4 ± 1.3	0.001	0.5 ± 1.0	< 0.001
Ferritin, ng/mL	64.1 ± 59.7	46.6 ± 50.1	< 0.001	− 17.4 ± 21.4	43.0 ± 43.1	43.1 ± 34.3	0.989	0.0 ± 23.9	0.004‡
Serum Fe, μg/dL	62.3 ± 40.8	50.6 ± 37.5	0.151	− 11.7 ± 49.9	59.6 ± 41.0	68.1 ± 37.4	0.217	8.5 ± 46.9	0.018
Transferrin, mg/dL	292.1 ± 95.1	261.3 ± 79.9	0.087	− 30.8 ± 109.4	306 ± 96.9	290.3 ± 87.8	0.363	− 15.7 ± 118.3	0.542
Transfer Saturation, %	20.4 ± 17.4	17.2 ± 14.1	0.311	− 3.2 ± 19.3	18.1 ± 14.1	20.9 ± 12.3	0.197	2.8 ± 14.9	0.088
TIBC, μmol/L	365.1 ± 118.9	326.7 ± 99.9	0.087	− 38.5 ± 136.7	382.4 ± 121.1	362.9 ± 109.7	0.364	− 19.6 ± 147.9	0.542
25OHD, ng/mL	20.7 ± 7.5	41.7 ± 17.2	< 0.001	21.0 ± 14.4	19.2 ± 8.1	44.0 ± 15.8	< 0.001	24.8 ± 14.6	0.239
ALPEM, ng/mL	6.7 ± 5.1	9.4 ± 21.1	0.334	2.8 ± 17.7	12.9 ± 19.0	12.8 ± 21.5	0.980	0.0 ± 8.2	0.198‡
ALPEA, IU/L	126 ± 4.54	128.5 ± 18.7	0.327	2.5 ± 15.6	131.5 ± 16.7	131.5 ± 19.0	0.968	0.0 ± 7.3	0.187‡
Osteocalcin, ng/mL	14.4 ± 17.3	29.6 ± 24.8	< 0.001	15.2 ± 11.7	17.7 ± 21.7	26.2 ± 25.8	< 0.001	8.5 ± 13.4	0.007
CTX, pg/mL	323.5 ± 419.8	597.1 ± 633.1	0.025	273.6 ± 734.3	642.1 ± 891.8	814.2 ± 973.1	0.099	172.1 ± 708.2	0.994‡
PTH, pg/mL	20.0 ± 19.3	22.0 ± 48.6	0.719	1.9 ± 33.2	36.2 ± 50.5	34.5 ± 56.4	0.561	− 1.8 ± 20.9	0.286‡
hsCRP, ng/mL	4753.0 ± 4112.1	3780.6 ± 3421.9	0.084	− 972.4 ± 3419.3	3588.1 ± 3664.9	3576.0 ± 3919.5	0.979	− 12.1 ± 3107.2	0.428‡
IL-6, pg/mL	21.0 ± 16.8	55.6 ± 53.7	0.001	34.5 ± 59.3	22.3 ± 25.6	34.4 ± 42.2	0.072	12.2 ± 45.8	0.033
TNF-α, pg/mL	23.1 ± 23.5	23.1 ± 33.3	0.992	0.0 ± 11.7	20.3 ± 14.2	20.0 ± 21.3	0.914	− 0.2 ± 14.8	0.300
TAC, mM	0.56 ± 0.07	0.55 ± 0.11	0.809	0.0 ± 0.1	0.52 ± 0.07	0.52 ± 0.09	0.867	0.0 ± 0.1	0.424‡
Malondialdehyde, μM	6.3 ± 3.0	5.5 ± 3.0	0.210	− 0.8 ± 4.0	6.1 ± 3.1	5.5 ± 3.2	0.158	− 0.7 ± 3.3	0.873
FBS, mg/dL	77.6 ± 15.3	97.6 ± 18.6	< 0.001	20.1 ± 16.9	79.2 ± 13.8	95.4 ± 12.0	< 0.001	16.2 ± 14.0	0.244
TG, mg/dL	139.7 ± 55.8	171.1 ± 82.7	0.004	31.4 ± 64.5	110.1 ± 49.8	118.7 ± 52.1	0.229	8.6 ± 48.7	0.116‡
TC, mg/dL	185.8 ± 30.8	182.8 ± 31.9	0.529	− 3.0 ± 29.5	166.9 ± 33.6	166.1 ± 33.0	0.784	− 0.9 ± 21.4	0.502‡
LDL, mg/dL	107.2 ± 21.0	97.3 ± 28.1	0.016	− 9.9 ± 24.6	94.0 ± 22.5	91.8 ± 22.6	0.287	− 2.2 ± 14.0	0.275‡
HDL, mg/dL	38.5 ± 7.0	41.6 ± 10.1	0.055	3.1 ± 9.7	43.6 ± 10.3	45.5 ± 9.2	0.041	1.9 ± 6.2	0.785‡

Data are presented as mean ± SD

RBC red blood cell, *Hb* hemoglobin, *Hct* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean cell hemoglobin concentration, *TIBC* total iron binding capacity, *ALPEM* alkaline phosphatase enzyme mass, *ALPEA* alkaline phosphatase enzyme activity, *CTX* C-terminal telopeptide, *PTH* parathyroid hormone, *hsCRP* highly sensitive C-reactive protein, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α, *TAC* total antioxidant capacity, *FBS* fasting blood sugar, *TG* triglyceride, *TC* total cholesterol, *LDL* low-density cholesterol, *HDL* high-density cholesterol

*P value for between group changes, obtained from Mann–Whitney or independent samples T test

† P value for within group changes, obtained from paired samples T tests or Wilcoxon signed rank test

Table 3 Habitual dietary intake of participants at baseline and after 12 weeks

Variables		Groups	
		D-Fe (mean ± SD)	D-P (mean ± SD)
Energy, kcal/day	Before	1786.3 ± 544.9	1837.2 ± 498.4
	After	1741.6 ± 382.6	1743.7 ± 566.9
	P value*	0.955	0.706
Carbohydrate, g/day	Before	234.7 ± 61.6	236.3 ± 52.1
	After	234.1 ± 58.3	226.7 ± 61.3
	P value*	0.759	0.500
Protein, g/day	Before	58.7 ± 20.8	57.9 ± 18.2
	After	57.1 ± 12.6	58.5 ± 14.1
	P value*	0.860	0.695
Fat, g/day	Before	75.8 ± 37.8	78.7 ± 34.7
	After	65.7 ± 30.7	74.2 ± 39.3
	P value*	0.283	0.413
Cholesterol, mg/day	Before	142.6 ± 1.9	172.7 ± 2.2
	After	138.8 ± 2.0	141.3 ± 2.2
	P value*	0.837	0.273
Dietary fiber, g/day	Before	10.8 ± 1.8	9.7 ± 1.6
	After	15.1 ± 1.4	11.6 ± 1.5
	P value*	0.087	0.094
Magnesium, mg/day	Before	206.9 ± 97.0	205.4 ± 99.6
	After	195.7 ± 73.7	192.2 ± 77.4
	P value*	0.834	0.850
Phosphorous, mg/day	Before	840.4 ± 363.1	810.3 ± 340.5
	After	848.6 ± 310.4	845.8 ± 291.5
	P value*	0.706	0.557
Iron, mg/day	Before	12.7 ± 4.3	14.1 ± 5.3
	After	12.0 ± 3.9	11.68 ± 4.7
	P value*	0.706	0.184
Calcium, mg/day	Before	538.7 ± 319.2	595.7 ± 272.5
	After	584.6 ± 217.3	613.4 ± 282.5
	P value*	0.209	0.902
Selenium, mg/day	Before	0.10 ± 0.06	0.10 ± 0.05
	After	0.13 ± 0.10	0.13 ± 0.03
	P value*	0.325	0.629
Vitamin K, μg/day	Before	102.8 ± 122.2	103.8 ± 156.8
	After	112.7 ± 133.7	97.5 ± 100.9
	P value*	0.727	0.482
Vitamin D, μg/day	Before	0.9 ± 1.1	1.2 ± 1.6
	After	0.9 ± 1.3	1.0 ± 1.4
	P value*	0.28	0.91
Vitamin E, mg/day	Before	4.0 ± 2.9	6.4 ± 4.5
	After	3.6 ± 3.3	8.5 ± 5.9
	P value*	0.082	0.112
Vitamin C, mg/day	Before	44.2 ± 2.9	47.2 ± 2.4
	After	62.3 ± 2.1	61.8 ± 2.2
	P value*	0.085	0.161

*Obtained from paired samples *T* test & Wilcoxon Signed Ranks Test

propeptide (bone formation marker) and an increase in PTH and tartrate-resistant acid phosphatase 5b (bone resorption markers) have been observed [34]. However, the level of 25OHD in this study did not change. Toxqui et al. observed a decrease in bone turnover markers and an increase in 25OHD when a group of women with iron deficiency anemia received an iron and vitamin D–fortified skimmed milk for 16 weeks [35]. A recent study demonstrated no change in serum levels of PTH and vitamin D binding protein after simultaneous supplementation of vitamin D and iron [36]. Recovery from iron deficiency anemia has been associated with lower bone remodeling without significant change in 25OHD level [14]. There are several differences between our study and similar trials that may justify our findings. These differences include the presence of iron deficiency in all participants and longer study duration. Additionally, in all of these studies, procollagen type I N-terminal propeptide was measured that is the most accurate marker of the bone formation [28]. However, $1,25(OH)_2$ D_3 induces mRNA, synthesis, and secretion of osteocalcin by human and rat bone cells in vitro which is sensitive and specific for the evaluation of formation in adults [37]. Unfortunately, none of these studies evaluated the effect of iron and vitamin D compared with vitamin D alone. Furthermore, we did not include a third group of iron-placebo in our trial. The increased bone turnover can be attributed to the coupling of formation and resorption. It has reported that serum osteocalcin is increased in patients with untreated osteomalacia [38]. So, it can be concluded that both groups experienced enhanced formation coupled with resorption. Additionally, calcium intake was less than recommended dietary intake in both groups. Low levels of calcium and phosphorus stimulate PTH secretion and consequent increased $1,25(OH)_2$ D_3 concentration [39]. Both hormones increase bone resorption to provide calcium and phosphorus required for calcification, and this could be another reason for the lack of decreased bone turnover in this trial. Four months supplementation with 20,000 IU/week vitamin D in 399 subjects with mean baseline 25OHD 34.0 nmol/L resulted in small but significant reduction of procollagen of type 1 amino-terminal propeptide (P1NP), without any effect on CTX, Dickkopf-1, sclerostin, TNF-α, osteoprotegerin, and receptor activator of nuclear factor κB ligand [40]. Those with high baseline PTH who had a decrease in PTH after the intervention, experienced higher decrease of P1NP and also significant reduction of serum CTX and increased sclerostin. Authors concluded that vitamin D supplementation is not effective or may even exaggerate bone loss if not combined with sufficient calcium intake. It is also seen that the response of bone turnover markers is different to osteoporosis treatments [41]; anti-resorptive treatments, like bisphosphonates, cause an early bone resorption and a delayed bone formation decrease, while anabolic treatments such as teriparatide result in an initial increase of bone formation markers and subsequent increase

Table 4 Sun exposure of participants at baseline and after 12 weeks

Variables			Groups		*P* value
			D-Fe (*n* (%))	D-P (*n* (%))	
Sun exposure duration	< 1 h/day	Before*	35 (85.3)	32 (84.2)	0.526
		After**	37 (90.2)	30 (78.9)	
	> 1 h/day	Before*	6 (14.7)	6 (15.8)	
		After**	4 (9.8)	8 (21.1)	
Time of sun exposure	10 am–3 pm	Before*	28 (68.2)	23 (60.5)	0.099
		After**	26 (63.4)	22 (57.8)	
	Other times	Before*	13 (31.8)	15 (39.5)	
		After**	15 (36.6)	16 (42.2)	
Sun exposure area	Face	Before*	14 (34.2)	15 (39.4)	0.264
		After**	16 (39.0)	17 (44.7)	
	Hands	Before*	5 (12.2)	1 (2.6)	
		After**	4 (9.7)	1 (2.6)	
	Face and hands	Before*	17 (41.5)	18 (47.5)	
		After**	15 (36.6)	16 (42.2)	
	Other areas	Before*	5 (12.1)	4 (10.5)	
		After**	6 (14.7)	4 (10.5)	
Sun screen use	Sometimes	Before*	34 (82.9)	31 (81.6)	0.670
		After**	35 (85.3)	31 (81.6)	
	Always	Before*	7 (17.1)	7 (18.4)	
		After**	6 (14.7)	7 (18.4)	
SPF of sunscreen	< 50	Before*	34 (82.9)	35 (92.1)	0.322
		After**	35 (85.3)	36 (94.7)	
	≥ 50	Before*	7 (17.1)	3 (7.9)	
		After**	6 (14.7)	2 (5.3)	

SPF sun protection factor

*Obtained from chi-squared test

of bone resorption. It seems that the action of vitamin D in our study was like an anabolic treatment.

Our study showed a significant increase of interleukin-6 in D-P group without any effect on in none of groups. Previous studies with vitamin D supplementation have shown conflicting results. A decline in CRP has been reported among patients in the intensive care unit after vitamin D supplementation [42], while supplementation with 100,000 IU/day vitamin D_2 or D_3 had no clinically meaningful effect on hsCRP level [43]. Monthly intake of 1.25 mg vitamin D for 2 years could not change inflammatory indexes like hsCRP, IL-6, IL-8, and IL-10 in 200 patients with osteoarthritis and vitamin D deficiency [44]. A meta-analysis on diabetic patients showed that vitamin D supplementation could reduce hsCRP but not TNF-α and IL-6 [45]. The difference in the results of studies could be due to supplementation dosage, treatment period, and the population studied. Higher serum concentration of hsCRP at baseline is effective on the results of vitamin D interventions [46]. Furthermore, supplemental vitamin D has increased interferon-γ and interleukin-10 in subjects that were vitamin D insufficient compared with sufficient healthy adults [47]. Perhaps lack of reduction of inflammatory markers in this study and similar studies is attributed to lower duration of intervention and recruiting healthy subjects, because most studies with positive results have been conducted in patients with inflammation [48]. Besides, observed increased interleukin-6 in the D-P group may be related to the lack of regulatory effect of vitamin D on some inflammatory markers, low concentrations of inflammatory markers at baseline, low dose of vitamin D, and a higher percentage of patients with vitamin D deficiency.

Antioxidant effects of vitamin D were first established by Wiseman in 1993 by the concept that vitamin D_3 and the active form $1,25(OH)_2$ D_3 halted iron-dependent peroxidation of liposomal lipid [49]. Vitamin D_3 mega dose of (200,000 IU) increased total antioxidant capacity in vitamin D–sufficient elderly women, and did not affect malondialdehyde [50]. After 12 weeks of consumption of vitamin D_3–fortified doogh compared with plain doogh, significant decrease of malondialdehyde and enhancement of glutathione and TAC were revealed [51]. Failing to show positive results in the present study may be related to differences in patients' genotype for vitamin D receptor, low-dose supplements required for reduction of lipid peroxidation, and insufficient time to impact on oxidative stress markers.

According to some observational studies on type 2 diabetes patients, vitamin D may have positive effects on glucose homeostasis [52, 53]. However, a systematic review found a weak correlation between vitamin D supplementation and descending fasting blood glucose and addressing insulin resistance challenge in patients with type 2 diabetes or impaired glucose tolerance, while no effect was seen in subjects with normal glucose tolerance [54]. In a recent systematic review and meta-analysis on 20 randomized clinical trials including 1464 patients with diabetic nephropathy, vitamin D supplementation had no impact on glycemic control indexes [55]. There are also some unpredictable findings. Supplementation with 50,000 IU/week vitamin D for 8 weeks in healthy adults > 65 years resulted in an increase in the number of people with insulin resistance from 13.2% to 36.8% [56]. The mean homeostatic model assessment of insulin resistance also increased from 1.39 ± 1.34 to 5.27 ± 3.72. Also, in Cox proportional hazard regression model, in contrast to multivariable binary logistic regression model, in order to predict the association between serum 25OHD level and the incidence of type 2 diabetes, a positive relationship was observed at the highest vitamin D quartile [57]. Here, an increase in FBS was revealed in our healthy young women that is inexplicable and should be evaluated in future studies. Short duration of the study and limited number of participants could confound our results.

After the intervention, a significant increase in TG and decrease in LDL were observed in the D-P group, while HDL increased significantly in the D-Fe group. In a meta-analysis (including 4 clinical trials, three of them with poor quality and high heterogeneity) to evaluate the effect of vitamin D supplementation on cardiometabolic risk factors in healthy adults, no significant effect was observed [58]. Some studies have reported a positive relationship between 25OHD and serum lipids [59, 60]. Ponda et al. observed that correcting vitamin D deficiency by administering 50,000 IU of vitamin D weekly during 8 weeks significantly increased LDL-cholesterol in vitamin D–deficient high cardiovascular-risk adults [61]. This controversy in results may be due to differences in study design, dose of vitamin, and participant's conditions. Furthermore, sufficient 25OHD has been attributed to better physical health and healthy lifestyle [62]. Our participants' vitamin D insufficiency may be related to non-specific chronic diseases. Supplementation with 50 mg iron with 500 mg docosahexaenoic acid or placebo in 76 women suffering from iron deficiency anemia caused significant decrease of apoAI in the group receiving iron and placebo [63]. Because of no significant between-group differences and small number of studies evaluating iron effects on cardiovascular health, it seems too early for conclusion.

The lack of effect of iron on vitamin D activation can be related to one or more of the following reasons: (a) the length of follow-up in studies evaluating bone markers is usually longer than the duration of the present study; (b) the dose of iron was not sufficient for iron status improvement; and (c) as previously mentioned, cytochrome P450 contains iron; however, in an animal study conducted by Dhur et al., in 1989 to assess the effects of different degrees of iron deficiency on cytochrome P450 enzymes, it was suggested that modification of iron-dependent enzymes may only happen after the third stage of iron deficiency [64]. A recent study in rats demonstrated negative effects of iron deficiency on renal 1α-hydroxylase activity and bone formation [65]. However, the authors concluded that the severity of iron deficiency anemia in their animal model is rare in human subjects. To the best of our knowledge, this is the first randomized, triple masked, clinical trial to assess the effect of vitamin D–iron co-supplementation, compared with vitamin D–placebo supplementation on bone health among healthy females. Strengths of the current study included the study methodology and adjustment of some confounding factors like physical activity level, sun exposure, age, and body composition. Unwanted significant increases of some metabolic and inflammatory biomarkers show a special need for conducting further studies regarding the effects of iron and vitamin D in individuals with concurrent vitamin d deficiency and iron deficiency anemia. Hence, further studies should be performed to explore in depth the effects of iron on vitamin D function and bone health.

This has some limitations which have to be pointed out. Firstly, all women were healthy and all parameters were within normal limits. It was assumed that most of participants with low hemoglobin levels are iron deficient; however, ferritin measurement did not prove our assumption and other reasons that can cause anemia may also affect the results of the present study. This study should have been performed in those with ferritin levels less than 30 ng/mL. We also did not assess factors like socioeconomic status which could affect our results. Finally, the present study was not statistically powerful enough to detect a difference between two groups in the primary outcome, 25(OH)D (~ 23%).

Conclusions

To sum up, we could not show additional effects of iron plus vitamin D intake on bone turnover, inflammatory, oxidative stress, and metabolic markers among healthy females with Hb ≤ 12.7 g/dL and 25OHD ≤ 29 ng/mL vitamin D.

Acknowledgments We thank the participants for their cooperation and participation in this study.

Authors' Contributions FA-Z and MV designed this study. BA, FA-Z, and HS participated in the conduct of the study. MS and FZ analyzed the data. BA, FA-Z, MS, and HS drafted the manuscript. MV and SMK critically revised the manuscript. All authors read and approved the final manuscript.

Funding This work was financially supported by the Vice Chancellor of Research, Iran University of Medical Sciences.

Data Availability The data can be made available on reasonable request to the corresponding author.

Compliance with Ethical Standards

Ethics Approval and Consent to Participate Written informed consent was obtained from all participants on recruitment. The protocol of this study was approved by the Medical Ethics Committee of Iran University of Medical Sciences, is in conformity with the Declaration of Helsinki (approval number: IR.IUMS.REC.1394.25971), and was registered at the Iranian Registry of Clinical Trials (IRCT registration number: IRCT201409082365N9) which is available at: http://irct.ir/user/trial/20288/view.

Consent for Publication All authors have given consent for the paper to be published by the corresponding author.

Competing Interests The authors declare that they have no conflict of interest.

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