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ORIGINAL ARTICLE

Adipose Tissue Inflammation and Oxidative Stress: the Ameliorative Effects of Vitamin D

Mahdieh Abbasalizad Farhangi,[1,5] Mehran Mesgari-Abbasi,[1] Ghazaleh Hajiluian,[2] Ghazaleh Nameni,[3] and Parviz Shahabi[4]

Abstract— Obesity is a low-grade inflammatory disease and is associated with numerous comorbidities. The current study was aimed to evaluate the effects of vitamin D administrations on markers of inflammation and oxidative stress in adipose tissue of high-fat diet-induced obese rats. In the beginning of the study, 40 rats were divided into two groups: normal diet and high-fat diet (HFD) for 16 weeks; then, each group was subdivided into two groups including ND, ND + vitamin D, HFD, and HFD + vitamin D. Vitamin D supplementation was done for 5 weeks at 500 IU/kg dosage. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, monocyte chemoattractant protein (MCP)-1, transforming growth factor (TGF)-β and IL-6 concentrations and markers of oxidative stress including glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT) concentrations in adipose tissue of rats were determined using ELISA kits and spectrophotometry methods, respectively. Vitamin D treatment led to a significant reduction in adipose tissue TNF-α concentrations in both ND + vitamin D and HFD + vitamin D groups ($P < 0.05$). Adipose tissue MCP-1 concentration also reduced in HFD + vitamin D group compared with HFD group. Among markers of oxidative stress in adipose tissue, SOD and GPx concentrations significantly increased in adipose tissue of HFD + vitamin D treated group compared with other groups ($P < 0.05$). Reduced food intake and weight gain was also occurred after vitamin D treatment. Vitamin D improved adipose tissue oxidative stress and inflammatory parameters in obese rats. Vitamin D treatment was also associated with decreased food intake and decreased weight gain in animals under a high-fat diet. Further studies are needed to better clarify the underlying mechanisms.

KEY WORDS: vitamin D; adipose tissue; oxidative stress; obesity; inflammation.

[1] Drug Applied Research Center, Nutrition Research Center, Department of Community Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Nutrition Research Center, Department of Community Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Neuroscience Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[5] To whom correspondence should be addressed at Drug Applied Research Center, Nutrition Research Center, Department of Community Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran. E-mail: abbasalizad_m@yahoo.com

INTRODUCTION

Obesity is a major health problem characterized by excessive fat deposition into adipocytes and non-adipose tissues, which is accompanied by a cluster of chronic metabolic disorders including cardiovascular diseases, diabetes, hypertension, dyslipidemia, and some types of cancers. In the past few decades, the prevalence of overweight and obesity has dramatically increased in both developed and developing countries. According to previous reports, more than 1.4 billion adults age of 20 years and

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older were overweight. Of these, over 200 million men and nearly 300 million women were obese [1–3]. Obesity and its related metabolic disorders are also linked to an overt oxidative stress and chronic inflammatory status. Oxidative stress along with a decline in antioxidant defenses causes an irreversible damage to macromolecules and a disruption in redox signaling mechanisms [4]. Obese individuals have demonstrated markers indicative of oxidative stress, including elevated measures of reactive oxygen species (ROS) [5] and diminished antioxidant defense, which is associated with lower antioxidant enzymes [6]. This oxidative status in obesity is closely associated with pro-inflammatory cytokines secretion, while these inflammatory cytokines can trigger the oxidative stress in a vicious circle [7]. Additionally, ingestion of lipid-reach meals can enhance oxidative stress and weight gain and facilitate the progression of insulin resistance [8]. Previous reports have demonstrated that adipose tissue oxidative stress is a major contributing factor in developing insulin resistance and diabetes [9]. Mature white adipose tissue cells and adipose tissue macrophages (ATMs) produce several inflammatory molecules like tumor necrosis factor (TNF) -α, interleukin 6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) contributing in development of insulin resistance [10]. Moreover, enhanced TNF-α concentration in obesity is closely related with increased TGF-β production in adipose tissue and studies have demonstrated that TGF-β gene expression is adipose tissue of obese mice is increased and is regulated by TNF-α production [11]. From histological point of view, the fat depots of obese animals are profusely infiltrated by macrophages—a state of low-grade chronic inflammation—which also contributes to the production of above-mentioned inflammatory cytokines in white adipose tissue (WAT) depots, especially in the visceral ones [12]. This low-grade inflammatory status and obesity-induced oxidative stress may be reversed by dietary changes and life style modifications; numerous studies have examined the efficacy of dietary interventions including caloric restrictions and change in macronutrient intakes in management of oxidative stress and inflammation in obesity [13]. Previous studies suggest that at least 10% reduction in body weight is necessary to reverse pro-inflammatory parameters which contribute to oxidative stress during obesity [14, 15]; however, this strategy of meaningful weight reduction may not be feasible or advisable to the general population. Among other dietary interventions, the potential role of vitamin D in management of obesity-induced oxidative stress has been suggested before as indicated that low-serum vitamin D status is associated with higher concentrations of oxidative stress markers and this condition may be worsened by TNF-α [16]. However, studies in this field are very scarce and limited. The antioxidant potential of vitamin D has been studied in several *in vivo* or *in vitro* studies. Vitamin D has been shown to downregulate intracellular adhesion molecule (ICAM)-1 expression in peripheral blood mononuclear cell following exposure to TNF-α [17] while serving as an antioxidant at the cellular membrane by decreasing lipid hydroperoxide [18, 19] and increasing total antioxidant status (TAS) and oxidative capacity in monocytes [20, 21]. Recently, Valcheva et al. has demonstrated that ROS production is enhanced in mice deficient for the vitamin D receptor [22].

Moreover, studies have demonstrated that obesity is associated with vitamin D deficiency and change in vitamin D homeostasis mostly because of increased sequestering of the steroid in adipose tissue [23]. It has been shown that for each 1 kg/m^2 increase in BMI, an estimated decrease of 0.74 nmol/L of vitamin D occurs [24]. More importantly, it has been shown that vitamin D inhibits TNF-α and IL-6 production by downregulating the nuclear factor (NF)-κB pathway [25, 26].

Considering the potential antiinflammatory and antioxidant roles of vitamin D and lack of evidence about the possible role of this vitamin in attenuating obesity-induced oxidative stress and inflammation in the adipose tissue, in the current study, we aimed to evaluate firstly that how high-fat diet induces inflammation and oxidative stress in adipose tissue of rats and secondly to examine the possible therapeutic roles of vitamin D administrations in ameliorating oxidative stress and inflammation in adipose tissue of these animals.

MATERIALS AND METHODS

The protocol of the study has been explained elsewhere [27]. Therefore, the procedure of the study is explained here briefly.

Animals

Forty male Wistar rats that weighted 200–220 g were purchased from the Pasteur institute animal care center (Karaj, Iran). The animals were housed five in each cage under standard conditions (light on from 07:00 a.m. to 07:00 p.m. and constant temperature of 25 ± 2 °C) with *ad libitum* access to food and water. Animal experiments were conducted in conformity with the National Institutes

of Health ethical guidelines for the care and use of laboratory animals (NIH; Publication No. 85–23, revised 1985) and approved by the veterinary ethics committee of the Tabriz university of medical sciences (Registration number: TBZMED.REC.1395.532). After a week of acclimatization and feeding a standard laboratory chow diet, rats were randomly assigned into two groups (n = 20, each group): either control diet or HFD. ND concluded 10% fat, 30% protein, and 60% carbohydrate and HFD with 59% fat, 11% protein, and 30% carbohydrate [25]. After 4 months of receiving ND and HFD, groups randomized in to two subgroups including ND, ND + vitamin D, HFD, and HFD + vitamin D. Vitamin D (Sigma-Adrich, USA) was administered in the dosage of 500 IU/kg/day by oral gavage alongside with the rats' prior diets for 5 weeks. The HFD and ND groups received Migliol (Sigma-Adrich, USA) as the placebo. Moreover, body weight was weekly measured by scale (PAND Industries, px3000, 5 kg ± 1 g) while food intake was monitored three times a week.

Preparation of Blood and Adipose Tissue Samples

After an overnight fasting, the rats were anesthetized with Ketamin (6.6 mg/kg) and Xylazine (0.3 mg/kg) intra peritoneally. Blood samples were obtained from cardiac puncture and centrifuged at 10,000×g at 4 °C for 20 min; sera were separated and stored in an ultra-low temp freezer (Jal Tajhiz Production, Iran) at −80 °C until assay. Finally, after rats were sacrificed by decapitation, their adipose tissues from abdominal regions such as epididymal (EAT), peri-renal (PAT), retroperitoneal (RAT), and mesenteric (MAT) were dissected and immediately stored at −80 °C until further use.

ELISA

Before and after vitamin D supplementation, serum measurement was performed to determine initial and terminal vitamin D level by individual enzyme-linked immunosorbent assay kit (ELISA) (Eastbiopharm, Zhejiang, China) according to the manufacturer's instructions. Adipose tissues samples were homogenized in phosphate buffered saline (PBS) and centrifuged at 10,000×g at 4 °C for 20 min, and clear supernatants were collected and the total protein concentration was measured by protein assay kit (Pars Azmun, Tehran, Karaj). TNF-α, MCP-1, IL-1β, IL-6, and TGF-β concentrations in the supernatants were determined using ELISA (Hangzhou Eastbiopharm, Zhejiang, China).

Measurement of Markers of Oxidative Stress in Adipose Tissue

The homogenates were used for determination of glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), and catalase by spectrophotometry, in accordance with the protocol provided with the assay kits.

Glutathione Peroxidase and Superoxide Dismutase Assessment

GPx activity was measured according to Paglia and Valentine [28] using Ransel, Randox Kit (UK). SOD was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced reduced nicotinamide adenine dinucleotide (NADH) oxidation according to Paoletti et al. [29] by using Ransod, Randox Kit (UK).

Malondialdehyde Assessment

MDA levels were measured using the thiobarbituric acid reactive substances (TBARS) method [30].

Catalase Assessment

The activities of CAT enzyme were measured according to Hugo Aebi method [31].

Statistical Analysis

All statistical analyses were performed using SPSS software, version 16. Kolmogorov–Smirnov test was performed for normality of the distributions of variables. Data are expressed as the mean ± SD. The data were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's tests and paired sample t test for comparisons between multiple groups and two groups. Repeated measures test was also used where $P < 0.05$ was considered as statistically significant.

RESULTS

Changes in Body Weight and Food Intake During the Study Period

The baseline body weights were similar among the different groups (Table 1). However, there was a significant difference in body weights of all treated groups at the end of the study (P = 0.001). Moreover, *post hoc* analysis showed that in intergroup comparisons of body weight, HFD led to a significant weight gain *versus* ND group

Table 1. Changes in Body Weight of Rats

Groups	1st week	16th week	21st week	*P* value[a]
ND	219 ± 10.83	276 ± 26.72	289 ± 29.80	*0.001*
ND + vitamin D	225 ± 22.09	278 ± 27.38	256 ± 26.90	*0.001*
HFD	219 ± 13.27	403 ± 4.13	425 ± 3.71	*0.001*
HFD + vitamin D	225 ± 13.77	340 ± 8.7	381 ± 7.80	*0.001*
P value[b]	0.69	*0.001*	*0.001*	

Data are expressed as means ± SD. Statistical differences between groups were assessed by one-way ANOVA followed by Tukey's test for *post hoc* analysis. Intragroup comparisons of body weight were performed by repeated measure analysis. $P < 0.05$ was considered as statistically significant
HFD high-fat diet, *ND* normal diet
[a] *P* value indicates intragroup difference
[b] *P* value indicates intergroup difference

($P = 0.001$). The food intake of studied groups has been presented in Fig. 1. As shown in this figure, vitamin D significantly reduced food intake in HFD-fed rats compared to control group ($P = 0.008$). In ND + vitamin D group *versus* ND group, food intake was not significantly decreased ($P = 0.54$).

Changes in Serum Vitamin D Concentrations During the Study Period

Baseline concentrations of vitamin D was not statistically significant between groups ($P = 0.50$). As expected, vitamin D administrations led to a marked increase in serum vitamin D concentrations in ND + vitamin D and HFD + vitamin D groups ($P = 0.001$), whereas serum vitamin D concentrations in HFD and ND groups reduced significantly at the end of interventional time period (Table 2).

Vitamin D Administration, Inflammatory Parameters, and Markers of Oxidative Stress in Adipose Tissue

Among inflammatory parameters (Figs. 2, 3, 4, 5, and 6), TNF-α concentrations in adipose tissue of ND group was significantly higher than ND + vitamin D group ($P < 0.05$). Moreover, TNF-α concentrations in adipose tissue of HFD group was significantly higher than HFD + vitamin D group ($P < 0.05$). MCP-1 in HFD + vitamin D group was significantly lower compared with HFD group. Other inflammatory parameters were not significantly different between study groups. Among markers of oxidative stress in adipose tissue, GPx and SOD activities in HFD + vitamin D group were significantly higher compared with ND and ND + vitamin D groups ($P < 0.05$; Figs. 7, 8, and 9). Catalase activity (Fig. 10) among HFD group was significantly higher compared with other groups.

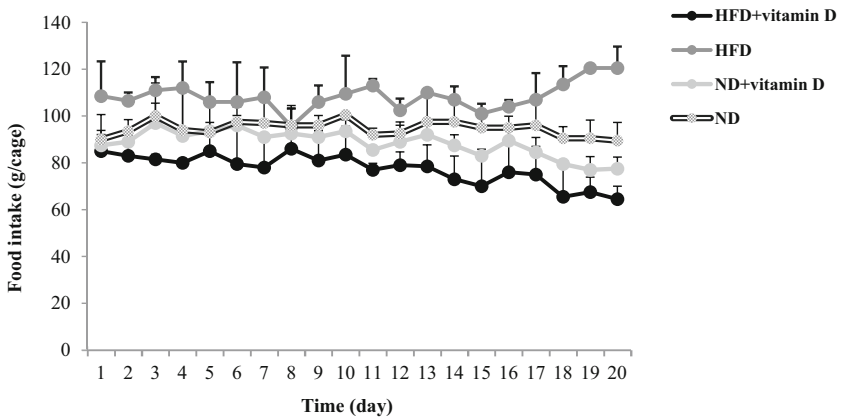

Fig. 1. Food intake of studied groups during study period. *HFD* high-fat diet, *ND* normal diet. A reduction in food intake after vitamin D administrations has been occurred in both vitamin D-administered groups ($P < 0.001$). Data are expressed as mean ± SD.

Table 2. Vitamin D Concentrations in Study Groups

Groups	16th week	21st week	Mean difference	*P* value[a]
ND	47.5 ± 7.32	36.3 ± 7.74	−11.20 ± 1	*0.001*
ND + vitamin D	54.9 ± 11.53	119 ± 26.01	64.2 ± 7.8	*0.001*
HFD	51.2 ± 11.16	37.7 ± 11.53	−13.5 ± 4.25	*0.01*
HFD + vitamin D	53.2 ± 14.11	124 ± 39.39	70.5 ± 12.85	*0.001*
P value[b]	0.50	*0.001*		

Data are expressed as means ± SD. Statistical differences between groups were assessed by one-way ANOVA followed by Tukey's test for *post hoc* analysis. Intragroup comparisons of vitamin D concentration was performed by paired *t* test analysis. $P < 0.05$ was considered as statistically significant
HFD high-fat diet, *ND* normal diet
[a] *P* value indicates intragroup difference
[b] *P* value indicates intergroup difference

Correlation Between Markers of Oxidative Stress and Insulin Resistance with Inflammatory Parameters

Table 3 presents the association between markers of oxidative stress and inflammatory biomarkers. These correlation matrix presents that GPx activity in adipose tissue was negatively associated with inflammatory parameters including IL-6, IL-1β, TNF-α, and TGF-β concentration, while MDA activity was positively associated with these inflammatory parameters. HOMA-IR was also in positive significant association with animals weight ($r = 0.65$, $P = 0.002$).

DISCUSSION

In the current study, vitamin D induced a significant decrease in TNF-α concentration of adipose tissue in ND + vitamin D *versus* ND group and in HFD +vitamin D *versus* HFD group. MCP-1 concentration in adipose tissue of HFD + vitamin D was significantly lower compared with HFD group. Additionally vitamin D increased the GPx activity in adipose tissue in HFD + vitamin D group *versus* ND and ND + vitamin D groups.

In the current study, feeding a high-fat diet for 16 weeks induced a meaningful weight gain among rats and this method was proved to be a useful model of putative effects of dietary fat in humans [32]. Also, it has been approved that rat models are useful tools for inducing obesity as they readily gain weigh when fed high-fat diets [33]. In our study, weight gain induced by high-fat diet was significantly more than that gained by those fed a normal diet. Final weight of animals in the current study was in positive association with index of insulin resistance.

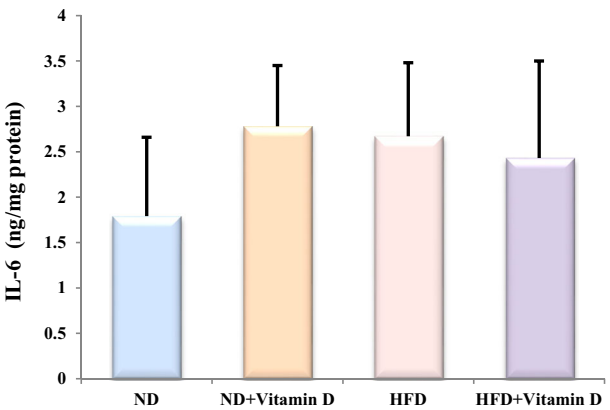

Fig. 2. Vitamin D administration and IL-6 concentration in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis. *Error bars* refer to the overall distribution of the data.

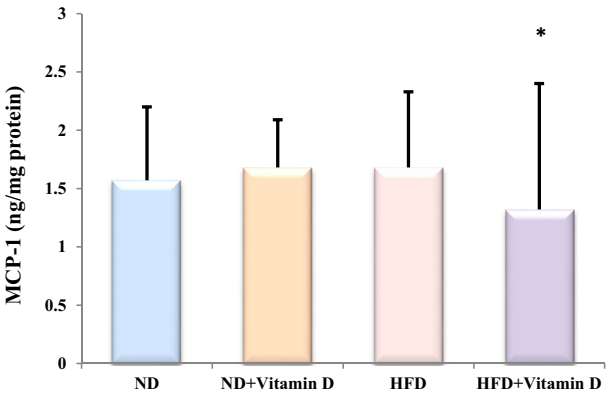

Fig. 3. Vitamin D administration and MCP-1 concentration in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis. (*$P < 0.05$ *versus* HFD). *Error bars* refer to the overall distribution of the data.

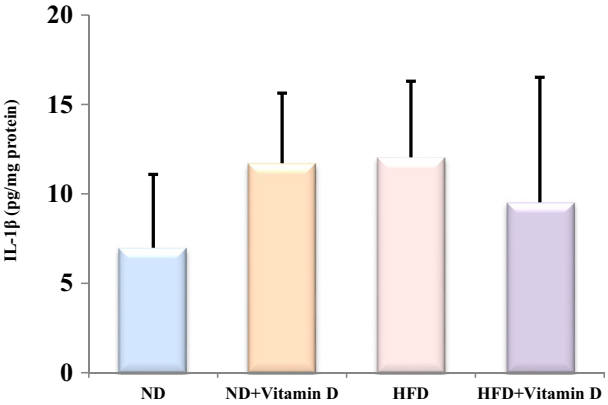

Fig. 4. Vitamin D administration and IL-1β concentrations in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis. *Error bars* refer to the overall distribution of the data.

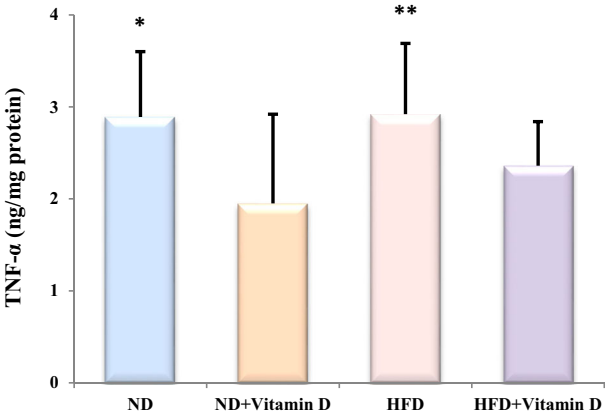

Fig. 6. Vitamin D administration and TNF α concentrations in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis (*$P < 0.05$ *versus* ND + vitamin D, **$P < 0.01$ *versus* HFD + vitamin D). *Error bars* refer to the overall distribution of the data.

This finding was in consistent with numerous previous reports of obesity-induced insulin resistance in animal models obtained by feeding high-fat diet most typically for 16 to 20 weeks [34–36]. It has been proposed that fat cells hyperplasia and fat deposition in mesentery are involved in change in metabolism and inducing insulin resistance by activating the Akt and mTOR signaling [36].

In the current study, vitamin D administration reduced TNF-α concentrations in adipose tissue of rats in both groups of ND + vitamin D and HFD + vitamin D. TNF-α, in WAT, is produced by mature adipocytes and by the stroma-vascular cells. Plasma TNF-α concentration is low even in obese animals, with no correlation with fat mass. However, there is a positive correlation between TNF-α expression and fat mass, indicating that TNF-α acts in a paracrine and autocrine manner in WAT depots [37]. TNF-α also increases lipolysis, with the concomitant increase in the plasma concentration of fatty acids; by the way, TNF-α leads to lipotoxicity and, indirectly,

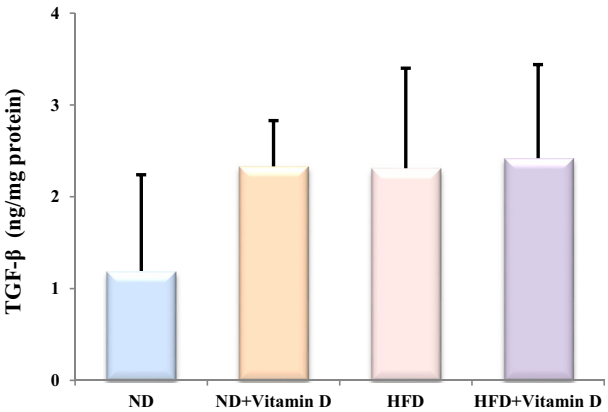

Fig. 5. Vitamin D administration and TGF-β concentrations in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis. *Error bars* refer to the overall distribution of the data.

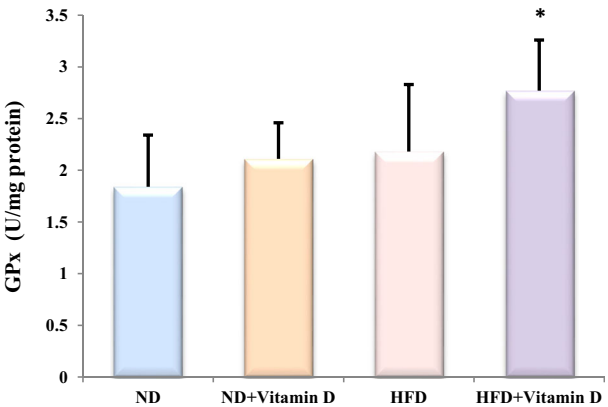

Fig. 7. Vitamin D administration and GPx activity in adipose tissue of studied groups. Data are expressed as mean ± SD statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis (*$P < 0.05$ *versus* ND, ND + Vitamin D). *Error bars* refer to the overall distribution of the data.

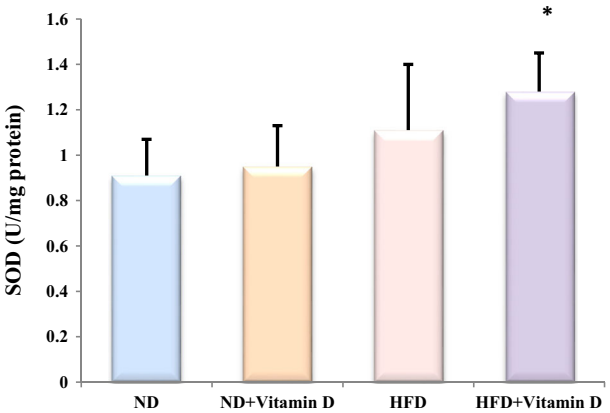

Fig. 8. Vitamin D administration and SOD activity in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis (*$P < 0.05$ *versus* ND, ND + vitamin D). *Error bars* refer to the overall distribution of the data.

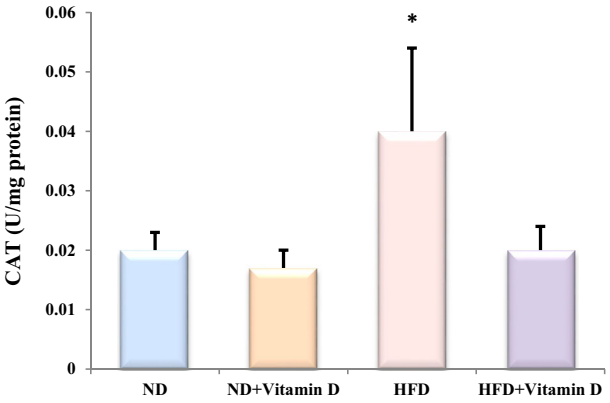

Fig. 10. Vitamin D administration and CAT activity in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis (* $P < 0.05$ *versus* ND, ND + vitamin D and HFD + vitamin D). *Error bars* refer to the overall distribution of the data.

insulin resistance [38]. TNF-α is also a potent regulator of oxidative stress. Elevated concentration of TNF-α has been shown to downregulate the expression of eNOS (diminishing the dilatory response) in human aortic endothelial cells [39] Specifically, TNF-α is a potent activator for activation of NADPH oxidase, resulting in the formation of ROS [40]. Picchi et al. [41] also examined the effects of TNF-α administration on oxidative stress response and reported higher O_2 levels and reduced NO bioavailability in Zucker obese rats compared to controls. In our study, adipose tissue TNF-α was negatively associated with GPx and positively associated with MDA concentrations; the role of adipose tissue TNF-α in progression of the tissue into oxidative status is mainly managed by the role of TNF-α in activation of the JNK or NFκB pathways; moreover, this association is bidirectional indicating that ROS production by itself is also a potent stimulator of TNF-α by adipose tissue [42].

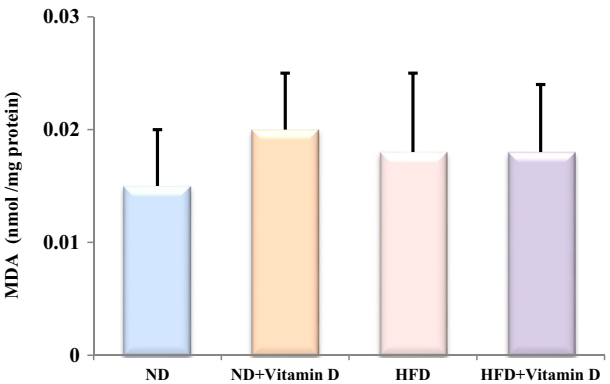

Fig. 9. Vitamin D administration and MDA concentrations in adipose tissue of studied groups. Data are expressed as mean ± SD. Statistical differences were analyzed using ANCOVA test adjusted for the confounding effects of food intake and weight of rats followed by Tukey's test for *post hoc* analysis. *Error bars* refer to the overall distribution of the data.

Table 3. Correlation Matrix Between Antioxidants and Inflammatory Factors in the Adipose Tissue

Parameter	*r* (*P*[a])	GPx	CAT	SOD	MDA	HOMA-IR
Weight	*r*	0.06	0.55	0.39	−0.048	0.65
	P	0.81	*0.018*	0.09	0.84	*0.002*
IL-6	*r*	−0.87	0.32	0.29	0.76	−0.002
	P	*<0.001*	0.18	0.22	*<0.001*	0.99
IL-1β	*r*	−0.87	0.45	0.16	0.69	0.21
	P	*<0.001*	0.06	0.48	*<0.001*	0.39
TNF-α	*r*	−0.88	−0.38	0.13	0.68	0.16
	P	*<0.001*	0.112	0.58	*0.001*	0.51
TGF-β	*r*	−0.79	0.34	0.06	0.73	0.11
	P	*<0.001*	0.15	0.99	*<0.001*	0.67
MCP-1	*r*	−0.79	0.34	0.25	0.65	0.065
	P	*<0.001*	0.15	0.28	*<0.001*	0.79

$P < 0.05$ was considered as statistically significant
IL interleukin, *TNF-α* tumor necrosis factor α, *TGF-β* transforming growth factor β, *MCP-1* monocyte chemoattractant protein 1
[a] *P* values obtained by Pearson correlation analysis

Vitamin D also reduced MCP-1 concentrations in adipose tissue of obese rats fed HFD + vitamin D while no effect in ND + vitamin D group has been observed. Monocyte chemoattractant protein-1 (MCP-1) is produced predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes [43]. Numerous studies have indicated the abundance of MCP-1 in both white adipose tissue and plasma of obese mice [44, 45]. Alongside with its positive strong association with other pro-inflammatory cytokines of adipose tissue in our study (data not shown), the MCP-1 concentrations are also in strong negative association with GPx and in positive association with MDA concentrations in adipose tissue indicating its involvement in oxidative stress as previously confirmed by several other reports [45, 46]. Similar associations were also observed for TGF-β and IL-1β concentrations in adipose tissue. We have previously reported the positive association between TGF-β and BMI in obese individuals [47, 48]. Although in the current report, no such a relationship was observed. The link between TGF-β and oxidative stress can be expressed in a vicious cycle; TGF-β1 increases ROS production and suppresses antioxidant enzymes, leading to a redox imbalance. ROS, in turn, induce/activate TGF-β1 and mediate many of TGF-β's fibrogenic effects [49, 50].

In conclusion, in the current study, vitamin D administration reduced inflammation and several oxidative stress markers in adipose tissue of high-fat diet-induced obese rats. Vitamin D also reduced weight and food intake in the high-fat diet-induced obese rats. Mechanistic studies are needed to better clarify several underlying etiologies.

ANOVA analysis of variance, *ATMs* adipose tissue macrophages, *GPx* glutathione peroxidase, *HFD* high-fat diet, *HOMA-IR* homeostatic model assessment of insulin resistance, *ICAM-1* intercellular adhesion molecule-1, *IL-1β* interleukin 1β, *IL-6* interleukin 6, *MCP-1* monocyte chemoattractant protein 1, *MDA* malondialdehyde, *ND* normal diet, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *TNF-α* tumor necrosis factor α, *TGF-β* transforming growth factor-β, *WAT* white adipose tissue

Authors' Contributions MAF was the main researcher who conceived and designed the project and wrote the draft of the manuscript and performed the statistical analysis, GH and GN and PSH were involved in the data collection and experimental lab works, and MMA performed the lab works.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that there is no conflict of interest.

Declarations Ethical Approval and Consent to Participate. Animal experiments were conducted in conformity with the National Institutes of Health ethical guidelines for the care and use of laboratory animals (NIH; Publication No. 85–23, revised 1985) and approved by the veterinary ethics committee of the Tabriz university of medical sciences (Registration number: TBZMED.REC.1395.532).

Consent to Publish. Not applicable.

Availability of Data and Materials. The raw data and other materials in the current study are available for all of the researchers throughout the world for scientific use with kind full permission.

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