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Research article

Dietary vitamin D ameliorates hepatic oxidative stress and inflammatory effects of diethylnitrosamine in rats

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ABSTRACT

The generation of reactive oxygen species (ROS) plays an essential role in the pathogenesis of several diseases. Its implication in inflammation has suggested a possible link between oxidative stress and activation/release of cytokines in precancerous states. Recent observational studies have suggested an association between inflammation and vitamin D deficiency; hence, suggesting that vitamin D could play a role in the pathogenesis of diseases. This study examined the antioxidant and anti-inflammatory potentials of vitamin D in diethylnitrosamine (DEN)-induced oxidative stress and inflammation in rats. Rats were divided into four experimental groups. While groups one and two were administered twice weekly with 30 mg/kg body weight DEN for six weeks, groups three and four were given normal saline. Groups one and three were fed with vitamin D deficient diet, while groups two and four were fed vitamin D diet during the experiment. After that, biomarkers of oxidative stress status were assayed spectrophotometrically. The concentration of inflammatory cytokines was determined using enzyme-linked immunosorbent assay (ELISA). DEN-induced vitamin D deficient diet group had increased antioxidant enzymes' activities. Also, there were elevated concentrations of thiobarbituric acid reactive substances (TBARS) and inflammatory cytokines in the same group. Vitamin D diet, however, reduced oxidative stress effects through the reduction in the activities of TBARS and caused a significant ($p < 0.05$) increase in nitric oxide concentration. Vitamin D diet significantly ($p < 0.05$) reduced the level of interleukin 1β and TNF-α produced in the deficiency state. These findings show that vitamin D may play an essential role in the regulation of hepatic oxidative stress and inflammatory responses.

1. Introduction

Nutrition plays a critical role in delaying the onset of various diseases, and it is used either as neoadjuvant or adjuvant therapy in the management of multiple chronic diseases [1], like arthritis, cardiovascular disease, diabetes, and cancer, which now account for 60% of all deaths [2]. Asides the burden, the morbidity of these diseases often result in substantial economic loss and social instability. Hence, changes in lifestyle, including diet, is a known cause of a continued rise in the global burden of these diseases. Consequently, several studies/reports have linked this to vitamin D deficiency or insufficiency [3, 4, 5]. Vitamin D is a fat-soluble steroid hormone whose primary function is the promotion of skeletal mineralization. It is now known to be involved in pro-apoptotic, anti-angiogenic, anti-proliferative, pro-differentiation, anti-invasive, and anti-metastatic biological processes [6, 7, 8, 9]. Additionally, there are also emerging pieces of evidence that vitamin D could have antioxidant and anti-inflammatory functions [10, 11, 12, 13].

Oxidative stress plays a significant role in activating various signaling pathways leading to inflammatory diseases and tissue damage [14]. Also, clinical conditions and continuous exposure to xenobiotics cause tissue injury and necrosis, which often result in inflammation [15]. The pathogenesis of chronic diseases, including cancer, resulted from continuous inflammation and tissue damage [16]. The tissue damage-induced inflammation resulting from an interplay between innate and adaptive immunity is characterized by persistent inflammatory stimuli and simultaneous release of inflammatory biomarkers, including cytokines and chemical mediators like Reactive Oxygen Species (ROS) [17]. Cellular response to xenobiotics, cytokines production, pathogenic attack, and mitochondrial oxidative metabolism produces ROS [18]. However, an array of antioxidant defense mechanisms scavenges these products, including hydroxyl radical ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) [18].

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Some of the defense mechanisms include superoxide dismutase (SOD) and glutathione-related enzymes. An imbalance in ROS produced compared with antioxidant activities leads to oxidative stress, a condition that is fundamental to the pathogenesis of chronic and degenerative diseases, like cancer [1, 19, 20, 21].

A ubiquitous example of oxidative stress-causing environmental toxicants is diethylnitrosamine (DEN). DEN is a tumorigenic compound found in tobacco, pharmaceutical products, and some agricultural chemicals [22, 23]. Hepatic detoxification by DEN involves the activation of cytochrome P450 enzymes in a process that generates ROS with concomitant oxidative stress [24, 25]. A report pointed to DEN as an oxidative stress-inducing agent in rodent tissues [26]. In particular, some studies provided details of oxidative stress as part of the molecular mechanisms involved in DEN-induced hepatocellular carcinoma [27, 28].

Furthermore, epidemic evidence, as well as experimental evidence, have linked vitamin D insufficiency to oxidative stress effects and inflammation, with all these playing a role in the prevention of conditions like diabetes, hepatic ischemia, and hepatorenal damage [11, 29, 30]. Hence, we hypothesized that dietary vitamin D could prevent DEN-induced hepatic oxidative stress and improve inflammation. This study, therefore, investigated the roles of vitamin D in DEN-induced hepatic inflammation by evaluating oxidative stress parameters and selected inflammatory cytokines.

2. Materials and methods

2.1. Chemicals/reagents

N-Nitrosodiethylamine ($C_3H_{10}N_2O$; >99%) used was purchased from TCI America, USA. Vitamin D mix (AIN 93-VX), vitamin D deficient mix (AIN 93), and mineral mix (AIN 93-M), used for the feed composition, were purchased from Dyets Inc. Bethlehem, PA, USA. Other chemicals used were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Experimental design

Covenant University Ethical Committee approved this study (CHREC/022/2019), with procedures done under the institutional animal care guidelines. Sixteen male albino Wistar Rats were divided into four experimental groups, which were housed with food and water ad libitum at room temperature with exposure to 12h dark/light. Animals were allowed to adapt for three weeks before the start of the experiment. Vitamin D deficient diet was given to groups 1 and 3, while groups 2 and 4 took a normal diet (with vitamin D) throughout the experiment. Animals were kept from direct sunlight and ultraviolet radiation during the experiment. The experimental groups with respective feeding regime as shown in Table 1 are;

Group 1: DEN-induced with vitamin D deficient diet (DEN/VDD)
Group 2: DEN-induced with normal diet (DEN/VD)
Group 3: Normal control with vitamin D deficient diet (NC/VDD)
Group 4: Normal control with normal diet (NC/VD)

Animals in groups 1 and 3 took 320 ng/kg body weight of paricalcitol (19-nor-1,25-dihydroxy vitamin D_2) [31] to deplete endogenous vitamin D. The baseline vitamin D concentration in treated groups was 7.74 ng/mL ± 0.822.

A week after the administration of paricalcitol, 30 mg/kg DEN was injected intraperitoneally twice weekly for six weeks with control groups administered the vehicle as described by Ding et al. [32]. After that, all rats were euthanized using 50 mg ketamine/5 mg xylazine.

2.3. Sample collection and processing

Blood samples were collected in heparinized tubes through the cardiac puncture and was separated to obtain plasma. The liver was removed, processed, and homogenized, as described by Rotimi et al. [33].

2.4. Histopathology

A section of the liver tissue was collected and fixed in 10% buffered formalin for histopathology. Samples were further processed using hematoxylin and eosin (H&E) staining. The congested sinusoids from the tissue slides were observed, thus showing noticeable red cells in between hepatocyte chords. Quantification of Liver injury was determined using a scoring system and classified based on the severity of the injury. No activity = 0, mild = 1, moderate = 2, and severe = 3.

2.5. Oxidative stress/antioxidant assays

Biomarkers of oxidative stress, including Superoxide dismutase (SOD), Glutathione S-transferase (GST), Glutathione (GSH), Peroxidase

Table 1. Diet composition (g/kg).

Composition	DEN/VDD NC/VDD	DEN/VD NC/VD
Maize starch	500	500
Soy Bean	350	350
Oil	50	50
AIN 93-VX AIN 93	- 10	10 -
Mineral mix	35	35
Fiber	50	50
Methionine	5	5

* **AIN 93-VX** include Thiamin HCl (0.6 g/kg), Riboflavin (0.6 g/kg), Pyridoxine HCl (0.7 g/kg), Niacin (3 g/kg), Calcium Pantothenate (1.6 g/kg), Folic Acid (0.2 g/kg), Biotin (0.02 g/kg), Cyanocobalamin (B12, 0.1%) (2.5 g/kg), Vitamin A Palmitate (500000 IU) (0.8 g/kg), Vitamin E Acetate (500 IU) (15 g/kg), Vitamin D3 (400000 IU) (0.25 g/kg), Vitamin K1 (7.5 g/kg), Sucrose (967.23 g/kg).

***AIN 93** include Thiamin HCl (0.6 g/kg), Riboflavin (0.6 g/kg), Pyridoxine HCl (0.7 g/kg), Niacin (3 g/kg), Calcium Pantothenate (1.6 g/kg), Folic Acid (0.2 g/kg), Biotin (0.02 g/kg), Cyanocobalamin (B12, 0.1%) (2.5 g/kg), Vitamin A Palmitate (500000 IU) (0.8 g/kg), Vitamin E Acetate (500 IU) (15 g/kg), Vitamin K1 (7.5 g/kg), Sucrose (967.48 g/kg).

***Minerals** include Calcium Carbonate (360 g/kg), Potassium Citrate.H_2O (30 g/kg), Potassium Phosphate, monobasic (252 g/kg), Sodium Chloride (74 g/kg), Potassium Sulfate (48.6 g/kg); Magnesium Oxide (26 g/kg), Ferric Citrate, U.S.P.(6.06 g/kg), Zinc Carbonate (2.65 g/kg), Manganous Carbonate (0.63 g/kg), Cupric Carbonate (0.3 g/kg), Potassium Iodate (0.01 g/kg), Sodium Selenate (0.01025 g/kg), Ammonium Paramolybdate.4H2O (0.00795 g/kg), Sodium Metasilicate.$9H_2O$ (1.45 g/kg), Chromium Potassium Sulfate.$12H_2O$ (0.275 g/kg), Lithium Chloride (0.0174 g/kg), Boric Acid (0.0815 g/kg), Sodium Fluoride (0.0635 g/kg), Nickel Carbonate (0.0318 g/kg), Ammonium Vanadate (0.0066 g/kg), Sucrose, finely powdered (209.806 g/kg).

(Px), Nitric oxide (NO), and Thiobarbituric acid reactive substances (TBARS), were assayed in the rat liver samples. TBARS level was tested using the method described by Buege and Aust [34]. SOD activity was determined as described by Marklund and Marklund [35]. The GST activity was assayed with the method of Habig et al. [36]. GSH concentration was tested using the method described by Ellman [37]. Griess method was used to determine NO level, as defined by Yucel et al. [38]. Px activity was determined using the method of Fergusson and Chance [39].

2.6. Inflammatory cytokines

Interleukins 6, 4, 10, 1β, and TNF-α were assayed in the plasma samples using ELISA. Kit for the test was obtained from Solarbio Science & Technology Co., Ltd. Beijing, China. The minimum detection limit of the ELISA kit ranged from 7-15 pg/Ml, while the coefficient of variation of both intra-assay and inter-assay was less than 10%.

2.7. Liver function parameters

Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total bilirubin (TB), Albumin (ALB), and Gamma Glutamyl transferase (ϒ-GT) were assayed spectrophotometrically in the plasma samples using Randox assay kits obtained from Randox laboratory ltd., UK.

2.8. Statistical analysis

R software (version 3.6.1) was used in analyzing the data with statistical significance explained using one-way Analysis of Variance (ANOVA). Post hoc analysis of the data was done using the Tukey HSD test. Results are presented as mean ± standard deviation (SD) with $P < 0.05$ considered to be significant.

3. Results

3.1. Sinusoidal congestion of the liver

In the histopathology result (Figure 1), the DEN/VDD group showed sinusoidal congestion and steatosis of the hepatic cells, which could lead to various forms of inflammation ranging from mild to severe [1, 2, 3]. The congestion effects were reduced with the administration of vitamin D diet (Figure 1b), where there was no congestion, but sinusoids were indicated. Both NC/VDD and NC/VD groups showed a healthy liver.

3.2. Vitamin D reduces oxidative stress in rat liver

The levels of lipid peroxidation, GSH, and NO, as well as the activities of Px, GST, and SOD, are shown in Figure 2. There was a significant TBARS increase ($p < 0.0001$) in DEN/VDD group as compared to the NC/VD (Figure 2c). Vitamin D diet, however, reduced lipid peroxidation significantly ($p = 0.0034$) in DEN/VD group (Figure 2c). DEN/VDD group showed a non-significant SOD increase ($p = 0.167$) as compared to the NC/VD group (Figure 2e). SOD level was reduced through vitamin D diet when compared to the DEN/VDD; however, it was also non-significant ($p = 0.18$). Furthermore, the level of GSH was non-significantly reduced in DEN/VDD group as compared to the NC/VD group ($p = 0.099$) (Figure 2b). Vitamin D diet had no significant effect on GSH ($p = 0.0996$) when compared to DEN/VDD group. Meanwhile, NO concentration in the DEN/VDD group was significantly ($p = 0.0006$) reduced as compared to the NC/VD group. When fed with vitamin D, there was a significant ($p = 0.0067$) increase in the hepatic NO concentration in DEN/VD, similar to

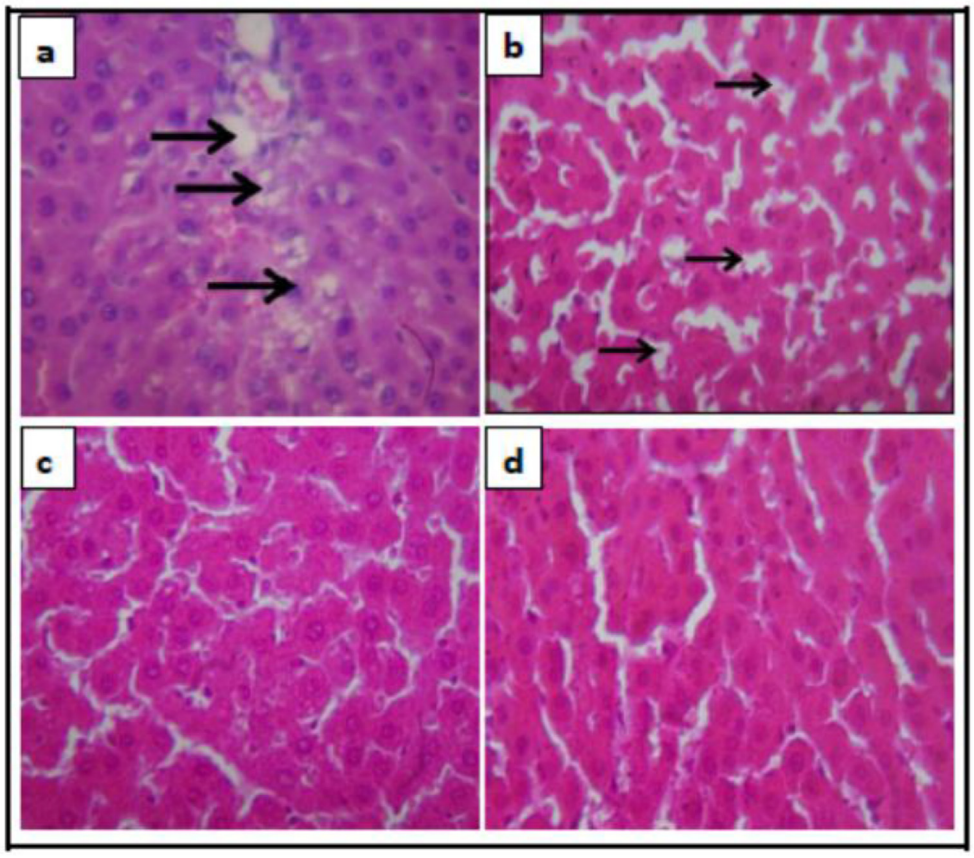

Figure 1. Histopathology of liver tissues of the four experimental groups; (a) DEN/VDD group showing sinusoidal congestion and steatosis (b) DEN/VD group showing sinusoidal congestion (c) NC/VDD group showing normal liver (d) NC/VD group showing normal liver.

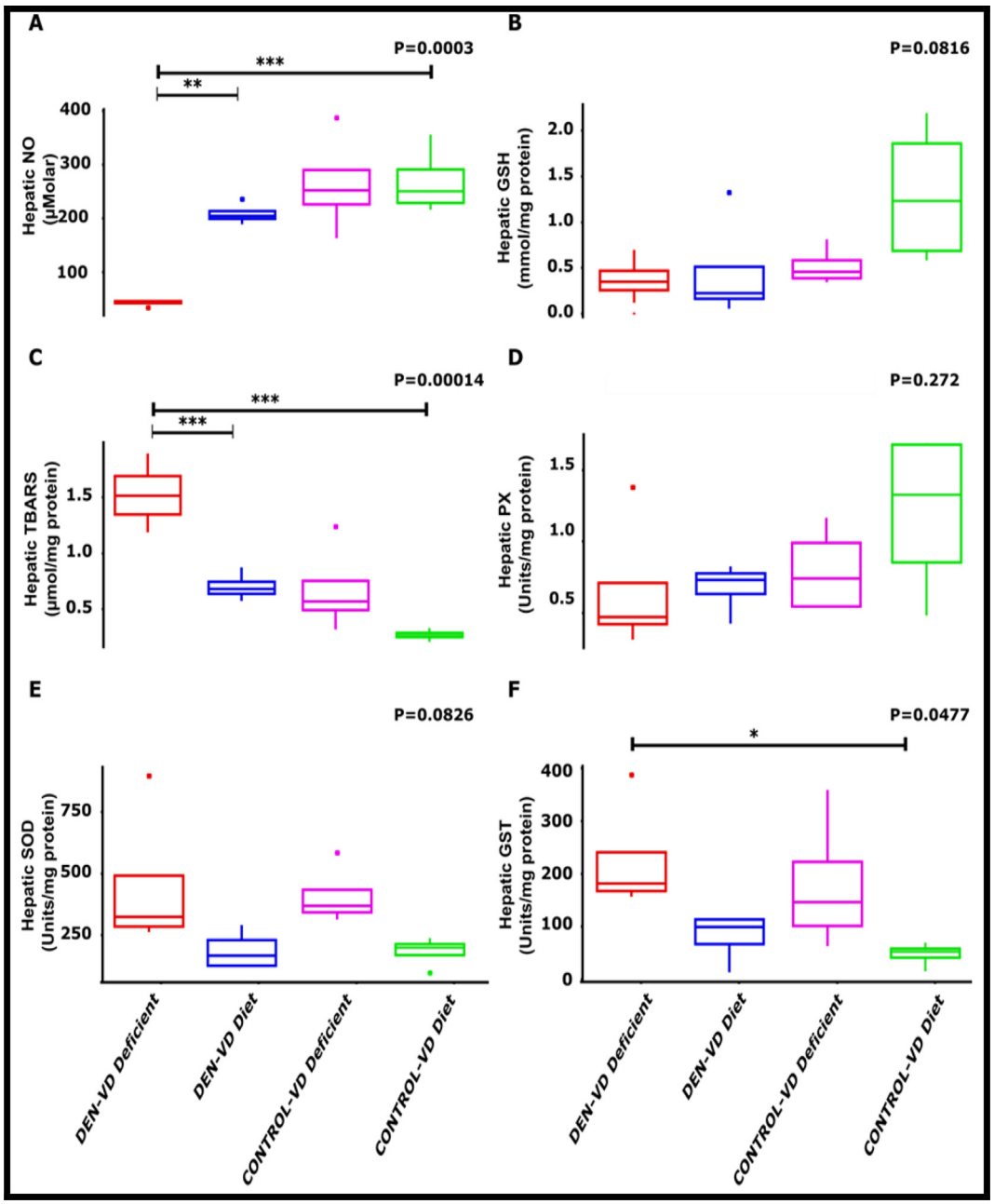

Figure 2. Effects of vitamin D on oxidative stress/Antioxidant parameters (a) Nitric Oxide concentration (b) Reduced glutathione level (c) Lipid peroxidation (d) Peroxidase activity (e) Superoxide dismutase activity (f). Glutathione S-transferase activity. Boxplots represent mean ± SD (n = 4). Significance: *** = <0.01; ** = 0.01–0.05; * = 0.05.

that of the NC/VD group (Figure 2a). The GST activity increased ($p = 0.059$) in the DEN/VDD group compared to the NC/VD (Figure 2f). Although our result showed a decrease with VD and DEN exposure, this reduction is, however, not significant ($p = 0.15$). Finally, vitamin D diet showed no significant difference ($p > 0.05$) on Px activity (Figure 2d).

3.3. Effects of vitamin D diet on liver function

AST activities were significantly increased ($p = 0.0316$) in the DEN/VDD group as compared to the levels in the NC/VD (Figure 3c). Although vitamin D diet had no significant effect ($p = 0.793$), there seems to be a slight reduction in the level of AST produced in DEN/VD. However, ALT levels significantly increased ($p = 0.0318$) in the DEN/VDD group to the NC/VD group, even though vitamin D diet had no significant effect on the increase (Figure 3d). The level of total bilirubin was non-significantly increased ($p = 0.0665$) in DEN/VDD in comparison to the NC/VD (Figure 3a). This increase, however, was not significantly reduced ($p = 0.946$) by vitamin D diet. A non-significant increase ($p = 0.166$) in the activities of ALP was shown in DEN/VDD as against the NC/VD group. This increase was also not significantly ($p = 0.505$) affected by the vitamin D diet, although, ALP level was interestingly upregulated (Figure 3e). In addition, no significant changes ($p > 0.05$) were shown in the levels of albumin and ϒ-GT activity when compared DEN/VDD to NC/VD (Figures 3b & 3f). Vitamin D diet also, do not have significant ($p > 0.05$) effects on these markers.

3.4. Vitamin D reduces the inflammatory effects of DEN

IL-1β, IL-10, TNF-α, and IL-4 concentration increased with DEN administration, as depicted in Figure 4. Significant increase in IL-1β ($p <$

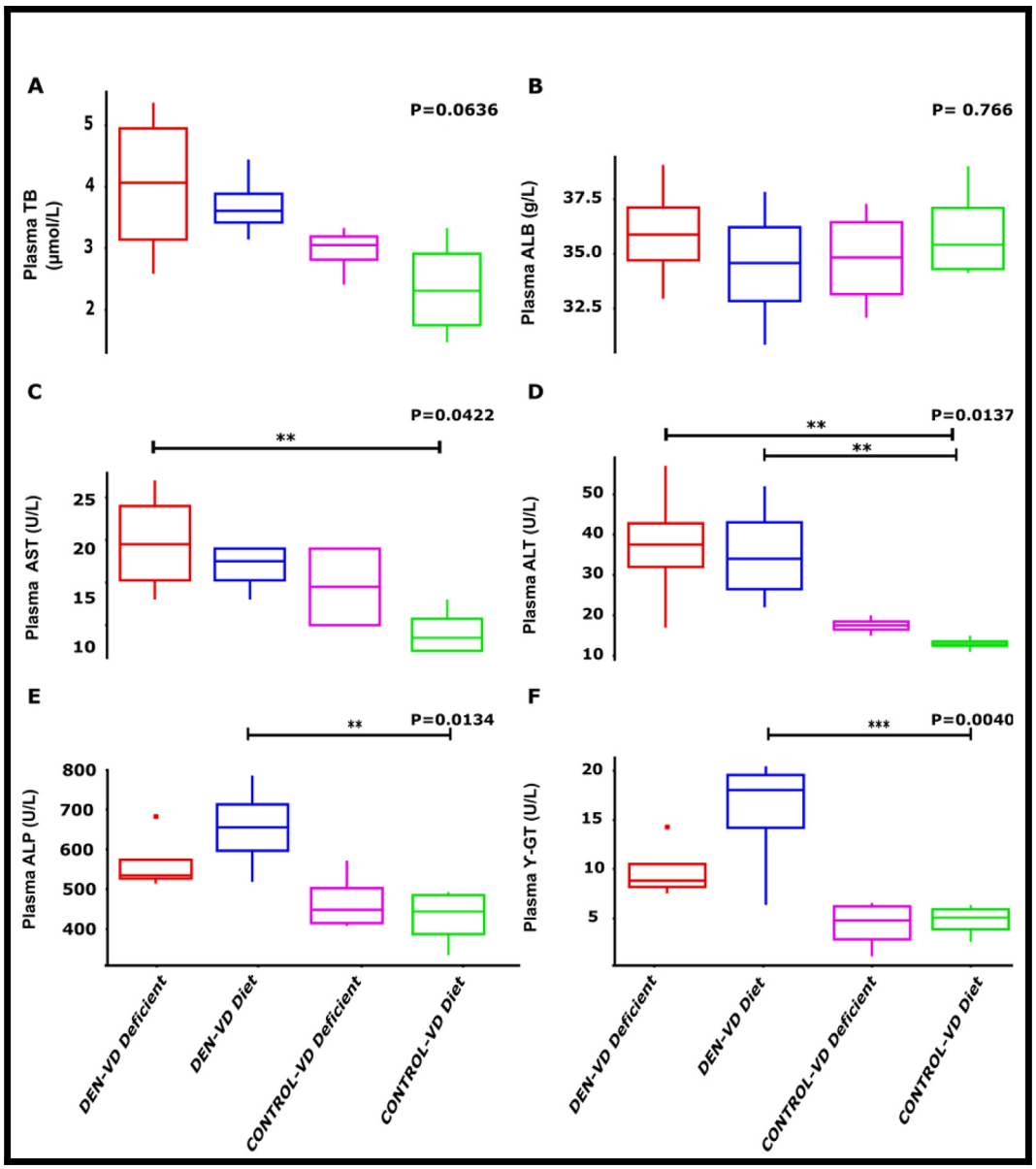

Figure 3. Effects of vitamin D on liver damage parameters (a) Total bilirubin (b) Albumin (c) Aspartate aminotransferase (d) Alanine aminotransferase (e) Alkaline phosphatase (f) Gamma Glutamyltransferase. Boxplots represent mean ± SD (n = 4). Significance: *** = <0.01; ** = 0.01–0.05; * = 0.05.

0.0001) was observed in the DEN/VDD group when compared to NC/VD group and vitamin D diet significantly ($p < 0.0001$) reduced this effect (Figure 4b). Also, IL-10 concentration was significantly increased ($p = 0.0008$) in the DEN/VDD group in relation to NC/VD group but was significantly decreased ($p = 0.0003$) with vitamin D diet (Figure 4d). In the same vein, TNF-α concentration, which was significantly increased ($p = <0.0001$) in the DEN/VDD group, was also reduced significantly ($p = 0.0061$) with the administration of vitamin D diet (Figure 4c). However, IL-4 concentration, which was significantly increased ($p = 0.0062$) in comparison to the NC/VD group were not significantly ($p = 0.923$) reduced with vitamin D diet (Figure 4e).

4. Discussion

DEN exposure increased the levels of oxidative stress biomarkers and inflammatory cytokines. These changes were characterized by an increase in TNF-α, IL-10, IL-4, IL-1β, lipid peroxidation, and GST activities and subsequent decrease in nitric oxide. These can be attributed to the hepatotoxic effect of DEN, thus, inducing oxidative stress and inflammation. At the end of the experiment, dietary vitamin D reduced the impact of some of these pathophysiological responses.

Increased SOD activity could increase the production of superoxide anion in the mitochondria. An essential function of SOD is to convert superoxide radicals to hydrogen peroxide (H_2O_2) since unneutralized superoxide radicals can cause the formation of hydroxyl radicals. The action and attack of hydroxyl radical on membrane phospholipids and triglycerides have been reported to be a cause of lipid peroxidation [40]. Increased lipid peroxidation is usually observed in different pathological states altering enzymes and inducing apoptosis [41], thus producing TBARS as by-products. From our study, the observed increased concentration of TBARS found in the DEN/VDD group can be attributed to an increase in oxidant production through the oxidative stress effect of DEN administered. Whereas the reduced nitrite concentration from nitric oxide observed can be linked to increased activity of antioxidant enzymes in the DEN/VDD exposed group. These changes were reduced in the DEN/VD group. The result was also similar to that observed in the NC/VD group. Nitric oxide's availability can be altered by the presence of ROS [42]. Generally, a decrease in nitrate concentration could be as a result of

L-arginine reduced availability and possibly that of other cofactors (e.g., tetrahydrobiopterin) [43]. The combination of these products results in the inactivation of nitric oxide production by uncoupling of endothelial nitric oxide synthase [43]. Another possible cause of reduced nitrite concentration linked to eNOS uncoupling is the S-glutathionylation of nitric oxide synthase, which concurrently increased superoxide production with increased SOD activity [44, 45]. These findings corroborate the importance of vitamin D in attenuating the production of TBARS, as shown in our study. Our result indicates the antioxidant ability of vitamin D, as described to inhibit 'iron-dependent lipid peroxidation in liposomes' [10].

In addition to various roles played in molecular and cellular processes in living organisms, the glutathione system is actively involved in defense against ROS. While GSH is important, GSTs are enzymes that detoxify both endogenous and exogenous toxicants into less active and more water-soluble products [46]. Increased oxidative stress and an imbalanced glutathione system have been associated with liver diseases [47, 48]. It is, therefore, of physiological importance to sustain the glutathione pool to reduce oxidative damage by ROS, especially during metabolic activities [49]. The reduction of GSH, as seen in previous reports [46] and a concurrent increase in GST activity has been reported to be a result of activation of adaptive mechanism since GSH serves as a substrate for GST. This effect indicates a possible way of combatting

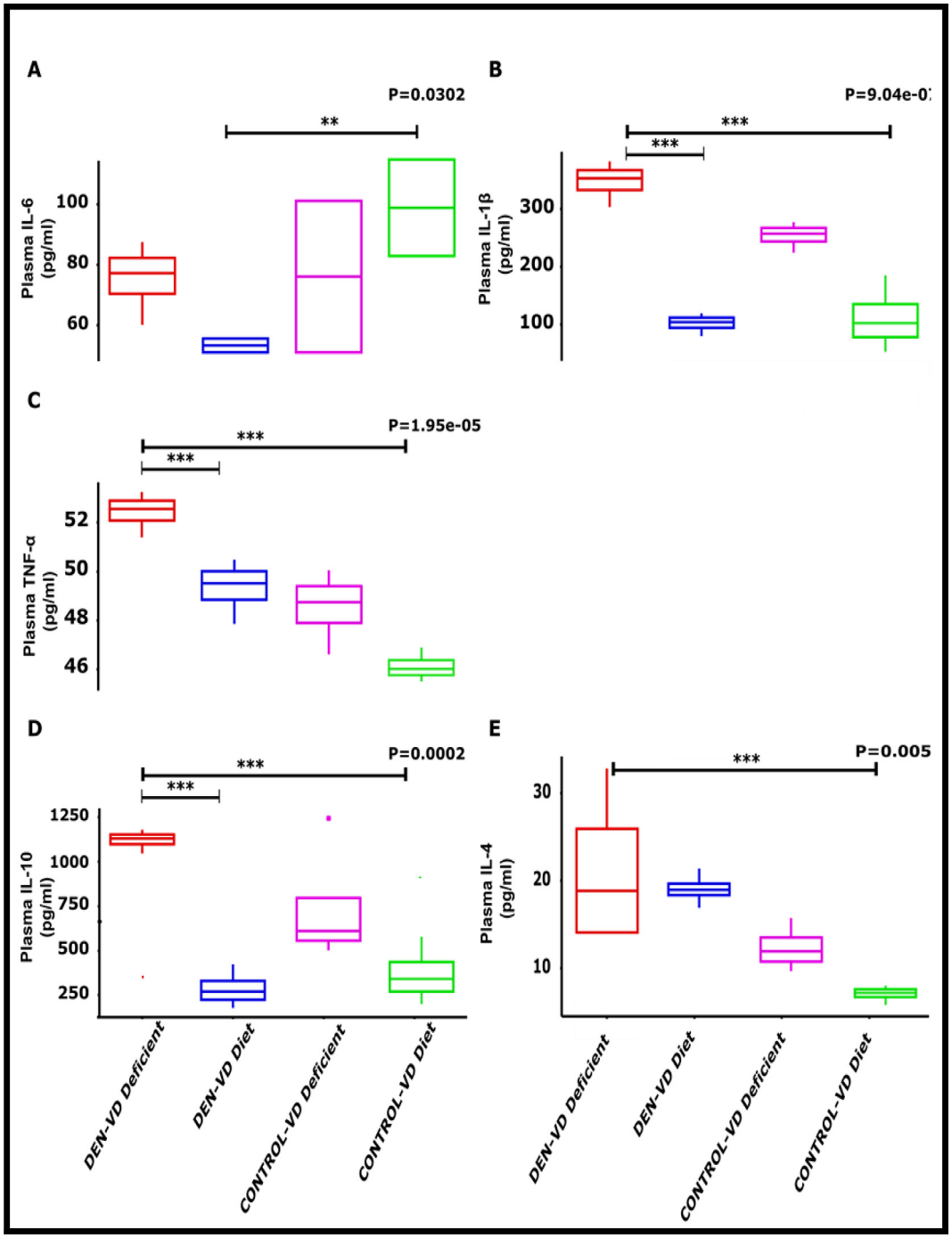

Figure 4. Effects of vitamin D on inflammatory cytokines (a) Interleukin-6 (b) Interleukin -1β (c) Tumor Necrosis Factor-α (d) Interleukin -10 (e) Interleukin -4. Boxplots represent mean ± SD (n = 4). Significance: *** = <0.01; ** = 0.01–0.05; * = 0.05.

oxidative stress through a corresponding increase in GST levels [46]. This idea was replicated in our study with a high GST level matched with decreased GSH levels in the DEN/VDD group.

The hepatotoxic effect of DEN in altering liver function enzyme activities has been established in the past [50, 51]. In the same vein, our results showed alterations in some of the parameters. Generally, a disruption of the cell membrane can lead to enzymes leaking into the cytosol [52]. The elevation of plasma activities of these enzymes is part of the diagnosis of liver damage or injury, which has recently been suggested to be caused by biliary cirrhosis, which eventually leads to cholestasis [53].

Consequently, cytokines are released as forms of immune response regulators in a bid to fight infections, trauma, and inflammation. Pro-inflammatory cytokines are known to upregulate inflammation, while anti-inflammatory cytokines act to down-regulate inflammation and hasten the healing process [54]. Activated macrophages are known to play essential roles in pathological pain by the action of pro-inflammatory cytokines they produce. In contrast, anti-inflammatory cytokines control the responses of the pro-inflammatory cytokines [55, 56]. In this study, we evaluated IL-1β, 6, and TNF-α as pro-inflammatory cytokines, while interleukin 4 and 10 were assessed as anti-inflammatory markers. The increased concentration of pro-inflammatory cytokines in TNF-α and IL-1β can be attributed to inflammation caused by DEN administration compared to the control group. High levels of anti-inflammatory markers observed in the DEN/VDD group demonstrate the possible role in controlling the pro-inflammatory cytokines produced in the same group. Our result is consistent with reports suggesting vitamin D has anti-inflammatory potentials by decreasing the elevated concentrations of inflammatory cytokines, including IL-1 and TNF-α, as shown in this study [57, 58, 59].

In conclusion, our study provided evidence that dietary vitamin D could attenuate the oxidative stress and inflammatory effects in the liver by reducing lipid peroxidation and cytokine production.

Declarations

Author contribution statement

I. Adelani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S.O. Rotimi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

E. Maduagwu: Conceived and designed the experiments.

E. Ogadi and C. Onuzulu: Performed the experiments.

O.A. Rotimi: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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