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Vitamin D_3 Protects against Cyclophosphamide-induced Neurotoxicity Via Modulation of Inflammatory Cytokines and Oxidative Stress Parameters

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ABSTRACT

Vitamin D_3's antioxidative properties make it potentially beneficial in many conditions where oxidative stress is implicated in the pathological processes. The study investigated the possible protective effects of Vitamin D_3 on weight loss, feed intake, brain oxidative status, markers of inflammation and cerebral cortex histomorphology in rats administered Cyclophosphamide (CYP). Sixty rats were randomly assigned into six groups (n=10). Groups A and D served as Normal and Cyclophosphamide control respectively and were fed standard rat chow, groups B and E received Vitamin D_3 (300 IU/kg), while those in groups C and F received Vitamin D_3 (600 IU/kg). Animals in group A-C received intraperitoneal normal saline on day1, while groups D-F got intraperitoneal Cyclophosphamide (100 mg/kg/day on day1). Standard diet and Vitamin D_3 supplementation were administered daily for 15 days. After the experimental period, animals were euthanised and blood was taken for the assessment of biochemical parameters. Cerebral cortex was also processed for histological study. Vitamin D_3 mitigated CYP-induced weight loss. It also improved total antioxidant capacity while reducing CYP-induced lipid peroxidation. Interleukin-10 increased, while the CYP-induced increase in TNF-α were mitigated following Vitamin D_3 supplementation. Again, protective efforts in cerebral cortex histomorphology were observed. The results showed Vitamin D_3's ability to protect against CYP-induced neurotoxicity in rats via modulation of inflammatory and oxidative stress parameters. It also highlighted Vitamin D_3's potential as a possible adjunct in cancer chemotherapy-induced tissue damage. However, further research will be needed to specify its exact role in cancer chemotherapy.

KEYWORDS: Secosteroids; Chemotherapy; Inflammation, Histomorphology; Neuroprotection; Oxidative stress

1. Introduction

Cyclophosphamide (CYP) is an important chemotherapeutic, and efficient immunosuppressive agent that belongs to the class of alkylating agents [1-3]. Cyclophosphamide's therapeutic usage has also gained widespread acceptance in bone marrow transplantation [4-5] and autoimmune conditions like rheumatoid arthritis [1], systemic lupus erythematosus (SLE) (including treatment for widespread proliferative lupus nephritis, modifications in neurologic and psychiatric manifestations, enhancement of platelet counts; improvement of gastrointestinal vasculitis, interstitial lung disease and diffuse alveolar haemorrhage) [6-11]. Cyclophosphamide has also been applied in managing certain renal conditions such as nephritic syndrome resistance to corticosteroid and focal segmental glomerulonephritis [12, 13], it is also effective in multiple sclerosis [3,14,15]. Other therapeutic applications include treating severe forms of other autoimmune diseases [1,11,16] and neuroblastoma [17,18].

Despite the significant pharmacological benefits of Cyclophosphamide, it has been associated with several adverse effects including neurotoxicity, bone marrow toxicity, bladder toxicity, cardiotoxicity, and gonadal damage among others, [19-24]. Though newer agents (platinum, taxanes, and targeted curatives) are sometimes used as substitutes for treating numerous solid tumours, cyclophosphamide is deemed relatively effective against many malignancies. This is often due to insufficient evidence for the superiority of the newer options over Cyclophosphamide usage [2]. However, the adverse effects associated with Cyclophosphamide have prompted researchers to hunt for adjuvants in form of drugs,

extracts, or supplements that have an ameliorative impact to mitigate these side effects.

Vitamin D is a fat-soluble secosteroid which is synthesised in two forms: Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol) [25]. Plants and fungi produce Vitamin D2 from ergosterol [26]; in contrast, human skin synthesises Vitamin D3 from 7-dehydrocholesterol (7-DHC) upon exposure to UVB radiation that comes from sunlight [27] with contributing efforts from the liver [28] and the kidney [29]. Vitamin D3 (1,25 (OH) 2D3) is primarily known to maintain normal calcium levels and bone development by regulating phospho-calcium metabolism [30]. Given that Vitamin D3 receptors (VDRs) are found in various tissues, including the prostate, brain, breast, pancreas, colon, and immune cells: it has been discovered to also play roles beyond bone health. Examples of such non-skeletal functions of Vitamin D3 are: regulation of arterial blood pressure, prevention of cardiovascular complications, enhancement of immunological responses, regulation of insulin production to prevent diabetes, protection against certain cancers, and renoprotection. Vitamin D3's application includes the management of multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and treatment-induced pain in cancer patients [31-33]. Also, approximately 3% of the human genome is regulated by Vitamin D3 [34]. Several tissues, including pancreatic islets, the prostate, colon, breast, macrophages, and vascular smooth muscle cells, contain 1-α-hydroxylase, enabling them to produce Vitamin D3 (1,25(OH)2D3) locally [35]. This local synthesis positions Vitamin D3 (1,25 (OH)2D3) to have a role in cancer prevention and innate/adaptive immunity modulation, [36,37] as well as brain health [38]. Evidence from epidemiology studies linked Vitamin D3 deficiency to increased risks of cancer, autoimmune diseases, diabetes, cardiovascular issues, and mental health disorders [39,40].

2. Materials and Methodology

2.1 Chemicals and Drugs

Vitamin D_3 tablets (1000IU) (Nature's field®), Cyclophosphamide injection (Endoxan Asta®), Normal saline.

2.2 Animals and Animal Welfare

Healthy Wistar rats used in this study were obtained from Empire breeders, Osogbo, Osun State, Nigeria. Rats were housed in wooden cages measuring 20 x 10 x 12 inches in temperature-controlled (22.5°C ±2.5°C) quarters with lights on at 7.00 am. Rats were allowed free access to food and water. All procedures were conducted in accordance with the approved protocols of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, within the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive (EU2010/63).

2.2 Experimental Methods

A total of sixty Wistar rats weighing 190 – 200g each were used for the study. The rats were randomly assigned into six groups (n=10 each). Group A (Control Group) received a single intraperitoneal dose of Normal Saline on the first day and distilled water orally for 15 days. Group B (Vitamin D_3 Control Group1) received oral 300IU/kg/day of Vitamin D_3 for 15 days. Group C (Vitamin D_3 Control Group2) received 600IU/kg/day of Vitamin D_3 (oral) for 15 days [41,42]. Group D (Cyclophosphamide Control Group) received a single intraperitoneal (i.p.) dose of 100mg/kg Cyclophosphamide at the start of the experiment [43]. Group E (Cyclophosphamide + Vitamin D_3 Group1) received a single i.p. dose of 100mg/kg Cyclophosphamide on the first day and 300IU/kg/day of oral Vitamin D_3 for 15 days. Group F (Cyclophosphamide + Vitamin D_3 Group2) received a single i.p. dose of 100mg/kg Cyclophosphamide on the first day and 600IU/kg/day of oral Vitamin D_3 for 15 days. At the end of the experimental period, animals were euthanised by cervical dislocation and blood was taken through an intra-cardiac puncture for the levels of inflammatory markers (IL-10, TNF-α), lipid peroxidation and antioxidant status. The brain was removed, observed grossly, weighed and either homogenised or fixed in 10 % buffered formol-calcium. Sections of the cerebral cortex were processed for paraffin embedding, cut at 5 µm and stained for histological study.

2.3 Determination of Body Weight and Feed Intake

Body weight and food intake were assessed weekly and daily respectively using an electronic weighing balance (Mettler Toledo Type BD6000, Greifensee, Switzerland) according to the protocol described [44]. Relative change in body weight or food intake was calculated for individual animals using the equation shown below, following which results for all animals were computed to find the statistical mean.

$$\frac{\text{Final body weight (or feed intake)} - \text{Initial body weight (or feed intake)}}{\text{Initial body weight (or feed intake)}}\ g$$

2.4 Biochemical Tests

2.4.1 Lipid Peroxidation

Lipid peroxidation level was measured as Malondialdehyde content as described previously [45-47]. Change in colour was measured using a spectrophotometer at 532 nm.

2.4.2. Antioxidant Status

Total antioxidant capacity was determined using commercially available assay kit. Colour changes were measured as described previously [48,49].

2.4.3 Tumour Necrosis Factor-α and Interleukin (IL) -10

Tumour necrosis factor-α and interleukin IL-10 were measured using enzyme-linked immunosorbent assay (ELISA) techniques with commercially available kits (Enzo Life Sciences Inc. NY, USA) designed to measure the 'total' (bound and unbound) amount of the respective cytokines as previously described [50].

2.5 Tissue Histology

Rat brains were dissected, fixed in neutral-buffered formol-calcium. The cerebral cortex was removed and processed for paraffin-embedding, cut at 5 µm and then stained with haematoxylin and eosin and cresyl fast violet for general histological study.

2.6 Photomicrography

Histologically processed sections of the cerebral cortex were examined microscopically using a Sellon Olympus trinocular microscope (XSZ-107E, China) with a digital camera, and photomicrographs were taken. Histopathological changes were assessed by a pathologist that was blind to the groupings.

2.7 Statistical Analysis

Data were analysed with Chris Rorden's ANOVA for windows (version 0.98). Data analysis was by One-way analysis of variance (ANOVA) and post-hoc test (Tukey HSD) was used for within and between group comparisons. Results were expressed as mean ± S.E.M. and $p < 0.05$ was taken as the accepted level of significant difference from control.

3 Results

3.1 Effect of Vitamin D_3 on Body Weight

Figure 1 shows the effect of vitamin D3 supplementation on percentage change in body weight in rats exposed to cyclophosphamide (CYP). There was a significant decrease in body weight in the CYP groups *CYP (-9.56%). CYP+VD300 (-3.33%) and CYP+ VD600 (-3.4%) compared to control (5.52%). Compared to CYP (-9.56%), there was a significant increase in body weight with CYP+VD300 (-3.33%) and CYP+ VD600 (-3.4%) respectively.

3.2 Effect of Vitamin D_3 on Feed Intake

Figure 2 shows the effect of vitamin D3 supplementation on percentage change in feed intake in rats exposed to CYP. There was a significant ($p < 0.05$) decrease in feed intake with CYP, CYP/VD 300 and 600 compared to control. Compared to CYP, feed intake increased with CYP/VD300 and CYP/VD600 respectively. Percentage change in feed intake decreased with CYP (2.17%). CYP+VD300 (4.02%) and CYP+VD600 (3.19%) as well as VD600 (5.93%) compared to control (12.61%). Compared to CYP (2.17%), feed intake increased with CYP+VD300 (4.02%) and CYP+VD600 (3.19%) respectively.

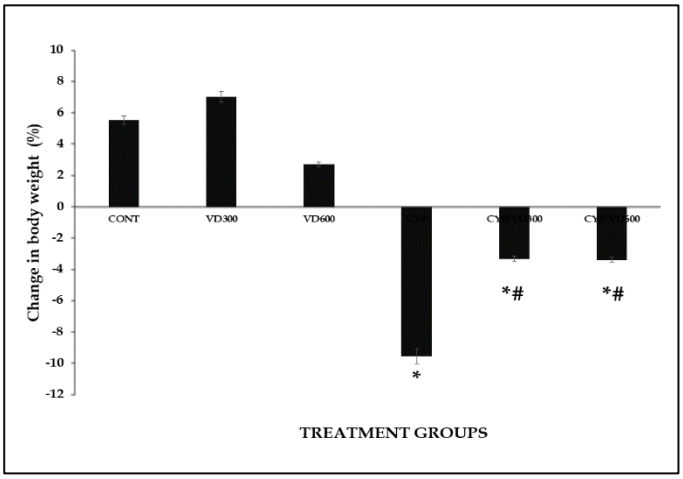

Figure 1: Effect of Vitamin D3 (VD) on change in body weight in CYP treated rats. Each bar represents Mean ± S.E.M, * $p < 0.05$ vs. control, #$p<0.05$ significant difference from CYP, number of rats per treatment group =10. CONT: Control; CYP: Cyclophosphamide, VD: Vitamin D_3.

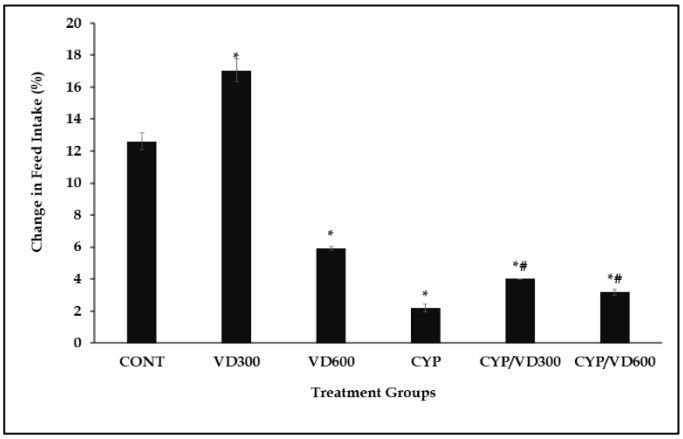

Figure 2: Effect of Vitamin D3 (VD) on relative change in feed intake in CYP treated rats. Each bar represents Mean ± S.E.M, * $p < 0.05$ vs. control, #$p<0.05$ significant difference from CYP, number of rats per treatment group =10. CONT: Control; CYP: Cyclophosphamide, VD: Vitamin D_3

3.3 Effect of Vitamin D_3 Supplementation on Oxidative Stress Parameters

Table 1 shows the effects of Vitamin D_3 supplementation on Total Antioxidant Capacity and Malondialdehyde levels in CYP-treated rats. Malondialdehyde (MDA) levels increased significantly with CYP, CYP/VD300 and CYP VD600 compared to control. Compared to CYP, MDA levels decreased

with CYP/VD300 and CYP/VD600. Total antioxidant capacity (TAC) increased significantly with VD300 and VD600 while it decreased significantly with CYP, CYP/VD300 and CYP/VD600 compared to control respectively. Compared to CYP, TAC increased with CYP/VD300 and CYP/VD600.

Table 1: Effect of Vitamin D_3 on Oxidative stress parameters

Groups	MDA (µmol/L)	TAC (mMTE)
Control	1.75 ± 0.02	4.58 ± 0.08
VD300	1.89 ± 0.39	5.04 ± 0.51
VD600	2.51 ± 0.17 *	5.23 ± 0.38
CYP	5.65 ± 0.3 *	2.06 ±0.15 *
CYP/VD300	2.13 ± 0.05 *#	5.03 ± 0.17 #
CYP/VD600	1.68 ± 0.14 #	4.51 ± 0.01 #

Mean ± S.E.M, * p < 0.05 vs. control, #p<0.05 significant difference from CYP, number of rats per treatment group =10. CYP: Cyclophosphamide; VD: Vitamin D_3; MDA: Malondialdehyde; TAC: Total Antioxidant Capacity.

3.4 Effect of Vitamin D_3 supplementation on Levels of Inflammatory Cytokines

Table 2 shows the effect of vitamin D_3 supplementation on inflammatory markers; tumour necrosis factor-α and interleukin-10 in CYP-treated rats. Interleukin-10 levels increased with VD300, VD600 and CYP/VD600 while it decreased significantly with CYP, but slightly with CYP/VD300 compared to control. Compared to CYP, interleukin-10 levels increased significantly with CYP/VD300 and CYP/VD600. Tumour necrosis factor α levels increased significantly with CYP and decreased with CYP/VD600 compared to control. Compared to CYP, tumour necrosis factor α levels decreased significantly with CYP/VD300 and CYP/VD600.

3.5 Effect of Vitamin D_3 supplementation on the cerebral cortex histomorphology

Figure 3 show representative haematoxylin and eosin (figure 3a-f); while, figure 4 shows cresyl fast violet (figure 4a-f) stained sections of the rat cerebral cortex. Examination of the haematoxylin and eosin-stained slides showed features of the cerebral cortex with evidence of numerous granule cells, pyramidal cells and neuroglia, in rats administered vehicle (Fig. 5a-c).

Multipolar shaped pyramidal cells with large, rounded, vesicular nucleus are seen scattered throughout the neuropil; granular neurons with large open-faced nuclei, prominent nucleoli and scant cytoplasm are likewise observed. The pink-staining background which is the neuropil is better appreciated in the cresyl fast violet-stained slides (figure 4a-c). In the group administered CYP, degenerating pyramidal cells with pale-staining nuclei, joined cells and granule cells are observed, these features are in keeping with neuronal injury. In groups supplemented with Vitamin D_3 (3d-e) was observed a protection against CYP-induced neuronal injury. Examination of cresyl-fast violet-stained sections of the cerebrum revealed well-delineated multipolar pyramidal cells, deeply-staining granule cells and neuroglia in the control group (Figure 4a) and groups administered Vitamin D_3 (Fig. 4b, 4c). However, degenerating pyramidal cells with pale staining nuclei were observed in the group administered CYP (Figure 4d), while a protection against neuronal injury was observed in the groups with Vitamin D_3 supplementation (Figure 4e, 4f).

Table 2: Effect of Vitamin D_3 on inflammatory cytokines

Groups	Interleukin-10 (pg/ml)	TNF-α(pg/ml)
Control	51.42 ± 14.59	515.25 ± 203.6
VD300	47.22 ± 12.67	473.42 ± 68.49
VD600	42.29 ± 2.86	372.01 ± 117.52
CYP	18.54 ± 1.8 *	982.01± 0.65 *
CYP/VD300	31.93 ± 8.95 #	536.46± 106.34 #
CYP/VD600	51.16 ± 1.51 #	491.02± 98.41 #

Mean ± S.E.M, * p < 0.05 vs. control, #p<0.05 significant difference from CYP, number of rats per treatment group =10. CYP: Cyclophosphamide; VD: Vitamin D3; IL-10: Interleukin-10; TNF-α: Tumour Necrosis Factor-α

4 Discussion

This study investigated the possible protective effects of vitamin D_3 supplementation on changes in biochemical and histomorphological markers of brain integrity, oxidant–antioxidant status, appetite, weight, and markers of inflammation in healthy rats administered Cyclophosphamide. The results showed that vitamin D_3 protected against Cyclophosphamide-induced changes in weight, feed consumption, antioxidant status, and inflammatory markers and cerebral morphology.

In this study, weekly body weight and percentage change in weight increased in the control group and decreased significantly in the groups administered Cyclophosphamide. The effects on body weight observed with Cyclophosphamide (CYP) in this study are consistent with the results of a number of other studies that had also reported that the administration of CYP caused significant weight loss [51]. On the other hand, treatment with vitamin D_3 mitigated CYP-induced weight loss. Vitamin D_3 is a secosteroid which plays a crucial role in

calcium homeostasis and bone health, but it has also been studied for its potential effects on body weight and metabolism [52-54]. Results of studies have varied significantly, with a few reporting vitamin D_3's abilities to mitigate weight gain [52], others observed no effect on body weight in normal sized animals [55] while other studies identified reduction in feed intake with Vitamin D_3 administration [56].

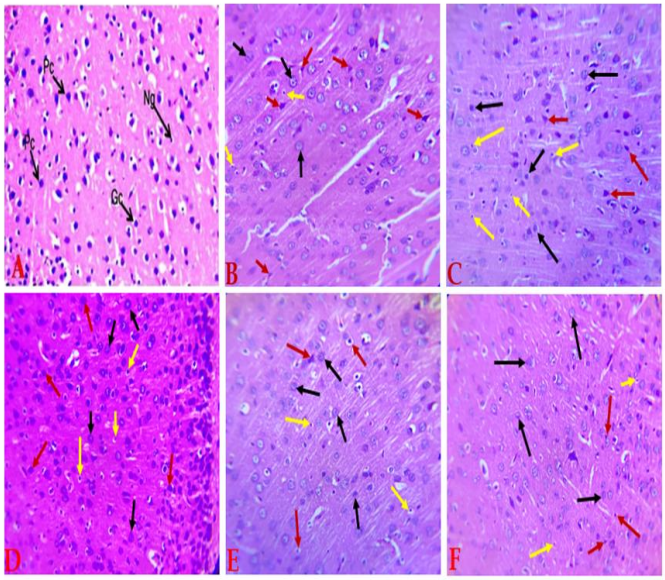

Figure 3: Photomicrographs of cerebral cortex sections stained with haematoxylin and eosin. Stained slides revealed distinct layers of the cerebral cortex with presence of numerous granule cells (Gc, black arrows), pyramidal cells (Pc, red arrows) and neuroglia (Ng, yellow arrows) in rats administered vehicle (a-c). Multipolar shaped pyramidal cells with large, rounded, vesicular nuclei are seen scattered throughout the neuropil; granular neurons with large open-faced nuclei, prominent nucleoli (more in D) and scant cytoplasm are also observed. **Mag. X 100.**

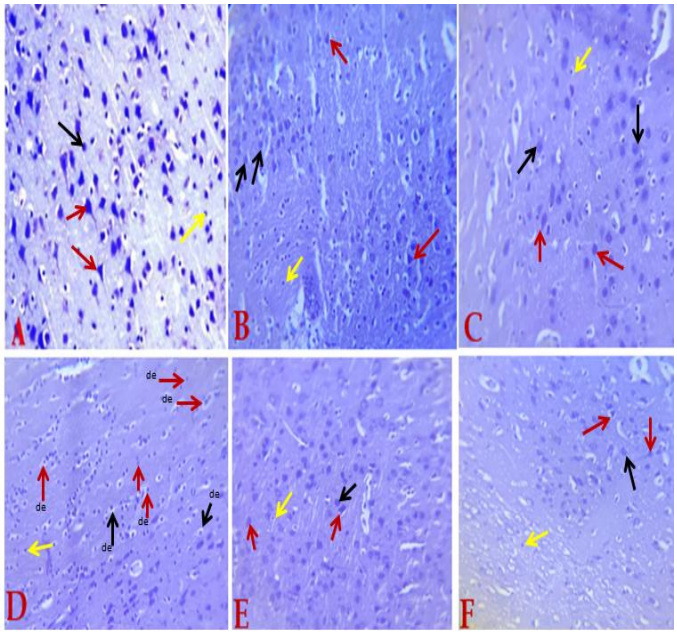

Figure 4: Photomicrographs of cerebral cortex-stained sections by cresyl fast violet. The pink-staining background which is the neuropil is better appreciated in the cresyl fast violet-stained slides (a-c). In the group administered CYP (d) numerous degenerating pyramidal and granule cells are observed, degenerating neurons are pale staining with shrunken pale staining nuclei, these features are in keeping with neuronal injury, numerous neuroglial are also seen as indication of inflammatory responses. In groups treated with vitamin D3 (e-f) was observed a protection against CYP-induced neuronal injury. **Mag. X 100.**

In this study, groups administered vitamin D_3 alone showed no significant difference in body weight from control, supporting studies that had reported such effects. This would suggest that vitamin D_3's effect on weight only came into effect in situations of body weight abnormalities like disease related weight loss or conditions of obesity. As also observed in this study (with CYP related weight loss), vitamin D_3's ability to reduce lipid peroxidation, increase antioxidant levels, and modulate the immune/inflammation system, via regulating the production of inflammatory cytokines and inhibiting the proliferation of proinflammatory cells [57-60] are possible mechanisms through which it reversed CYP-induced weight loss. The administration of CYP in this study was associated with a significant reduction in feed intake while treatment with vitamin D_3 generally mitigated CYP induced decrease in feed intake. In groups administered vitamin D_3 alone, no significant difference in feed intake was observed, supporting earlier observations that vitamin D_3's effect on the body was to restore normalcy. A few studies have also reported that vitamin D_3 did not increase muscle strength or power, energy consumption, resting metabolic rate and body composition [61]. In the CYP groups, vitamin D_3 supplementation reduced CYP induced decrease in feed intake and weight loss, consistent with the results of other studies that had revealed that lack of vitamin D_3 receptor (VDR) led to a lean phenotype with reduced body weight, decreased mass of white adipose tissue, promoted rate of β-oxidation, and enhanced energy expenditure leading to weight loss [62]; which Vitamin D_3 supplementation could potentially prevent, according to this study. Vitamin D_3's effects on body weight and feed intake can also be attributed to its ability to influence central processes that regulate energy homeostasis [61,63]. There have also been reports that vitamin D_3 can scavenge superoxide, hydroxyl, peroxyl and alkoxyl radicals in tissues [59, 64], leading to a decrease in lipid peroxidation level and an increase in the level of the total antioxidant capacity. Studies have continued to report the benefits of vitamin D_3 in modulating inflammatory activity and in protecting against brain injury as well as that of other organs [58-66]. The beneficial effects of vitamin D_3 could be attributed to a decrease in proinflammatory cytokine (TNF-α) [67]; while levels of anti-inflammatory cytokines (IL-10) increased [68] similar to this study. The neuroprotective effects of vitamin D_3 have also been linked to its ability to inhibit the formation of malondialdehyde, scavenge reactive oxygen species. There have been reports that the ability of Cyclophosphamide to cross the blood brain barrier and induce oxidative stress is one of the many ways through which it induces neurotoxicity [69]. Also,

in this study, Cyclophosphamide was associated with neuronal injury and an increase in migrating neuroglial cells, a significant indicator of cerebral inflammation, as previously reported [70] following Cyclophosphamide administration. Vitamin D_3's ability to protect against and reduce brain injury has been reported severally [65,71,66,72]. While most studies have examined its benefits in dementia and Alzheimer's disease [73-75], only a few studies have examined its potential benefits in chemotherapy induced neurotoxicity [76]; hence this study serves as an eye-opener to more potential effects of Vitamin D_3 in chemotherapy induced neurotoxicity.

Conclusion

Vitamin D_3 supplementation causes significant dose-related modifications in antioxidative stress markers (malondialdehyde, total antioxidant capacity) and inflammatory cytokines (tumor necrosis factor- α (TNF-α), and interleukin-10) in cyclophosphamide-induced neurotoxicity in rats. Considering its ever-increasing potential clinical applications and natural sources, these study findings would assist in making objective appraisals of the protective effect of vitamin D_3 in the course of Cyclophosphamide chemotherapy.

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Availability of data and materials

Data generated during and analysed during the course of this study are available from the corresponding author on request.

Declaration of ethical approval

Ethical approval for this study was granted by the Ethical Committee of the Faculty of Basic Medical Sciences ERC/FBMS/038/2024

Competing interests

None

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