RESEARCH ARTICLE

Liposome-based Nanoparticles Encapsulating Vitamin D3 Attenuate IL-6 and TNF-α in a Menopausal Mouse Model

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Abstract

BACKGROUND: Vitamin D3 is an essential regulator of immune function, however its bioavailability is limited. Liposomes as nanocarriers can enhance vitamin D3 absorption and delivery, however the application of liposomal vitamin D3 in postmenopausal remains underexplored, particularly in preclinical models. Estrogen deficiency during menopause promotes immune dysregulation and elevates proinflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α. This study was conducted to evaluate the effects of liposomal vitamin D3 supplementation on serum vitamin D3, IL-6, and TNF-α levels in an ovariectomy-induced menopausal mouse model.

METHODS: Mice were randomly divided into four groups comprising non-surgical control (N), ovariectomized without treatment (D−), conventional vitamin D3-treated (D+), and liposomal vitamin D3-treated (LD). Treatments were administered daily via oral gavage for two months. Serum vitamin D3, IL-6, and TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA). IL-6 and TNF-α data were analyzed by ANOVA with Duncan's post-hoc test, while vitamin D3 data were analyzed using the Brown-Forsythe test with Games-Howell post-hoc test ($p<0.01$).

RESULTS: Ovariectomy significantly decreased vitamin D3 levels and increased IL-6 and TNF-α levels in the D− group. Conventional vitamin D3 supplementation (D+) significantly decreased serum vitamin D3 levels and slightly decreased IL-6 and TNF-α levels. Liposomal vitamin D3 (LD3) significantly increased vitamin D3 levels and decreased TNF-α, only slightly decreasing IL-6. Correlation analysis showed a negative association between serum vitamin D3 levels and both cytokines.

CONCLUSION: Administration of vitamin D3 liposomes was able to increase vitamin D3 levels and suppress IL-6 and TNF-α towards normal levels. LD3 offers enhanced bioavailability and anti-inflammatory effects, making it a promising therapeutic strategy for managing menopause-associated inflammation and related systemic disorders.

KEYWORDS: menopause, liposomal VD3, inflammation, IL-6, TNF-α

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Introduction

Menopause is characterized by a decline in ovarian estrogen production, leading to reduced estrogen levels that cause thinning of the epithelium, loss of vaginal mucosal folds, and ultimately vaginal atrophy.(1) Estrogen deficiency is also a major risk factor for osteoporosis, a chronic degenerative disease prevalent among postmenopausal women.(2) This prolonged menopausal period is further associated with multiple physiological changes, including age-related immune decline. Immunosenescence affects several systems endocrine, nervous, cardiovascular, gastrointestinal, and musculoskeletal contributing to increased susceptibility to infections, reduced vaccine responsiveness, a higher prevalence of autoimmune disorders, and an elevated risk of chronic diseases such as cancer.(3) Moreover, reduced estrogen levels impair cellular proliferation in the vaginal

epithelium and negatively impact vitamin D metabolism, thereby compounding the health risks associated with menopause.(1,4)

The synthesis of calcitriol [1,25(OH)$_2$D], the active form of vitamin D , in the skin and kidneys declines with age, accompanied by reduced intestinal absorption. This absorption is mediated not only by passive diffusion but also by active transport mechanisms involving cholesterol transporters.(4,5) Estrogen regulates the expression of these transporters, and its decline during menopause may therefore impair vitamin D uptake. Epidemiological studies have consistently documented a high prevalence of vitamin D deficiency in postmenopausal populations. For example, a study in India reported that 53.35% of postmenopausal women were vitamin D deficient (6), while a large European cohort revealed that 77.4% of 21,236 postmenopausal women had serum 25(OH)D concentrations below 30 ng/mL.(7)

Hypovitaminosis D, commonly observed among postmenopausal women, has been linked to impaired immunity and elevated systemic inflammation.(8) Several cytokines, including interleukin (IL)-1 (9), IL-6 (10-12), and tumor necrosis factor (TNF)-α (11,12), are closely associated with inflammatory processes. Vitamin D deficiency correlates with increased levels of proinflammatory cytokines such as IL-6 and TNF-α, both of which play pivotal roles in the pathogenesis of osteoporosis. Osteoporosis is a chronic metabolic bone disease characterized by reduced bone mass and deterioration of bone microarchitecture, leading to an increased risk of fractures. The primary mechanism underlying bone loss in osteoporosis involves enhanced bone resorption mediated by cytokines such as receptor activator of nuclear factor kappa-B ligand (RANKL), which stimulates osteoclast differentiation and activity. (13) Notably, vitamin D supplementation has been shown to decrease IL-6 and TNF-α levels while improving bone health outcomes in postmenopausal women.(14,15)

Adequate vitamin D intake (2000–4000 IU/day) is recommended to prevent bone loss and support musculoskeletal health in peri- and postmenopausal women. (16) In postmenopausal populations, a daily dose of 2000 IU has been reported to be effective for correcting vitamin D deficiency and maintaining adequate serum concentrations with continued therapy.(17) Beyond its skeletal effects, vitamin D exerts important immunomodulatory functions by enhancing T-cell activation through upregulation of cluster of differentiation (CD)4, CD8, and CD69 expression. It also possesses both anti-inflammatory and antiproliferative properties.(18) There are two primary forms of vitamin D: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). As a secosteroid hormone, vitamin D plays a pivotal role in regulating immune responses.(19)

However, the bioavailability of vitamin D is limited due to its lipophilic nature and variable absorption, which can be influenced by factors such as food matrix composition, age, gastrointestinal health, and genetic polymorphisms. (20) Liposomal formulations have emerged as promising nanocarriers to enhance vitamin D delivery. Liposomes are capable of encapsulating hydrophobic compounds, thereby improving their stability, enabling controlled release, and prolonging circulation time.(21) Despite these advantages, the application of liposomal vitamin D3 in postmenopausal women remains underexplored, particularly in preclinical models. Therefore, this study investigates the immunomodulatory and therapeutic potential of liposomal vitamin D3 supplementation in an ovariectomized mouse model of menopause. The findings are expected to support the development of targeted interventions for managing immunological and skeletal complications in postmenopausal women.

Methods

Animal Maintenance and Ovariectomy Procedure

Experimental mice were sourced from PUSVETMA Surabaya and all procedures were approved by the Ethics Committee of the Faculty of Medicine (No.11/EC/KEPK/FKUA/2025). The animals were housed at the Universitas Airlangga animal facility, where they underwent a three-month acclimatization period to achieve a body weight of 25–30 g prior to surgery. Ovariectomy was conducted under general anesthesia induced by intramuscular administration of 0.1 mL xylazine–ketamine. Under aseptic conditions, a midline abdominal incision was made, the ovaries carefully excised, and the incision closed with catgut sutures. Wound powder was applied to promote healing and minimize the risk of infection. A recovery period of 2–3 weeks was provided to ensure full recovery before animals were included in subsequent experimental procedures.

Animal Treatment

One month after ovariectomy, a vaginal swab was collected. Mice showing a diestrus phase in their estrous cycle were considered ready for inclusion in the study (Figure 2). The animals were then randomly allocated into four groups: non-surgical control (N), ovariectomized without treatment (D−), ovariectomized plus conventional vitamin D3 (D+),

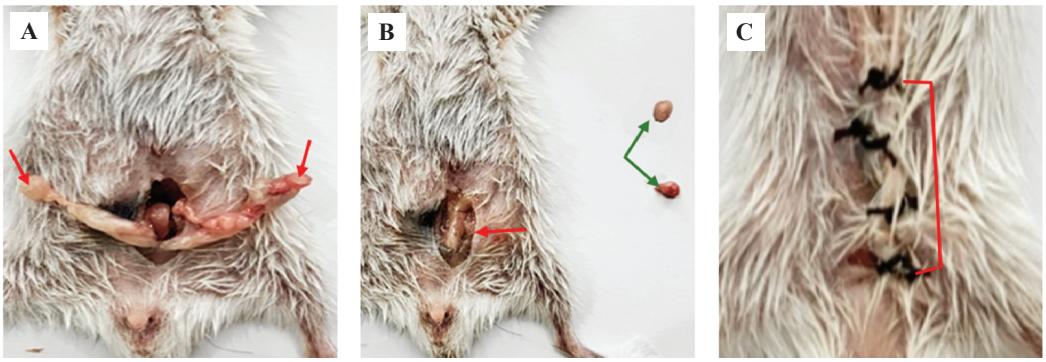

Figure 1. Ovariectomy procedure in mice. A: The uterus was exteriorized and the ovaries were excised (red arrows). B: The uterus was returned to the peritoneal cavity (red arrows), while the ovaries were removed (green arrows). C: The inner and outer skin is sutured, disinfected, and treated with antibiotics (red arrows).

and ovariectomized plus liposomal vitamin D3 (LD3). The N group did not undergo ovariectomy and received coconut oil as a vehicle control. The D− group underwent ovariectomy and received only coconut oil, while the D+ group received 2000 IU of standard vitamin D3 following ovariectomy. The LD3 group received 2000 IU of LD3. Treatments were administered daily for two months via oral gavage (Figure 3). All interventions were performed under standardized conditions to ensure consistency and reliability of experimental outcomes.

Measurement of Vitamin D3, IL-6, and TNF-α Levels Using the Enzyme-linked Immunosorbent Assay (ELISA) Method

After 90 days of treatment, blood samples were collected via cardiac puncture under appropriate anesthesia. The samples were allowed to clot at room temperature and subsequently centrifuged at 3000 rpm for 10 min to isolate the serum, which was then stored at −80 °C until analysis.

Serum levels of IL-6, TNF-α, and vitamin D3 were quantified using the Sandwich ELISA kit. IL-6 was measured with a mouse IL-6 ELISA kit (Cat. No. E0049Mo; BT Laboratory, Shanghai, China), TNF-α with a mouse TNF-α ELISA kit (Cat. No. E0117Mo; BT Laboratory), and vitamin D3 with a mouse 25-hydroxyvitamin D3 [25(OH) D3] ELISA kit (Cat. No. EA0066Mo; BT Laboratory). For each assay, 40 µL of serum sample was loaded into pre-coated wells, followed by 10 µL of biotin-labeled antibody and 50 µL of streptavidin-HRP. Plates were incubated at 37°C for 60 minutes and then washed five times with washing buffer. Subsequently, 50 µL of substrate solution A and 50 µL of substrate solution B were added, and color development occurred in proportion to the analyte concentration. The reaction was terminated by adding 50 µL of stop solution, and absorbance was measured at 450 nm. IL-6, TNF-α, and vitamin D3 concentrations were determined from standard curves generated for each analyte.

Data Analysis

Statistical analysis was performed using SPSS software version 25 (IBM Corporation, Armonk, NY, USA). Data are presented as mean±standard deviation (SD). Each experimental group included seven biological replicates (n=7) to ensure reliability and reproducibility. The differences in IL-6 and TNF-α levels between groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences in vitamin D3 levels between groups were assessed using the Brown-

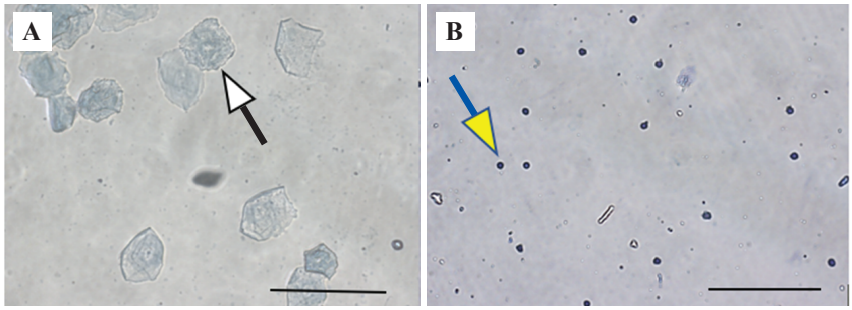

Figure 2. Vaginal smear histology. A: Estrus phase in non-surgical mice, characterized by abundant cornified epithelial cells (white arrowheads). B: Diestrus phase in ovariectomized mice one month after surgery, characterized by numerous leukocytes (yellow arrowheads). Black bar: 100 µm.

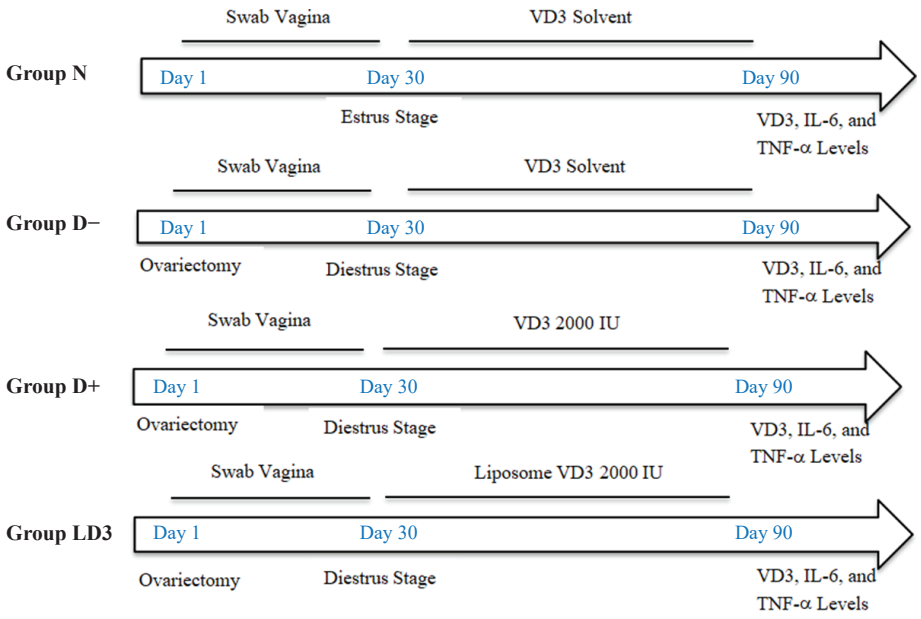

Figure 3. Experimental schedule. On day-1, mice were allocated into four groups: N, D−, D+, and LD3. Ovariectomy was performed in the D−, D+, and LD groups, and its success was confirmed by vaginal smear cytology showing the diestrus phase. On day-30, daily treatments were initiated for two months: groups N and D− received coconut oil as the vehicle, group D+ received 2000 IU of conventional VD3, and group LD received 2000 IU of liposomal VD3. On day-90, intracardiac blood sampling was performed, and serum was isolated for measurement of VD3, IL-6, and TNF-α levels.

Forsythe test, followed by the Games-Howell post-hoc test. Statistically significant differences were marked with asterisks *(p<0.01) in the corresponding figures.

Results

Increased Vitamin D3 Levels after Treatment with LD3

The non-surgical group exhibited the highest vitamin D3 concentration, reflecting baseline physiological levels in the absence of hormonal disruption. In contrast, the group that underwent ovariectomy without vitamin D3 supplementation (D–) showed a significant decrease in serum vitamin D3 levels (p<0.01, Brown–Forsythe test and Games–Howell post hoc) compared with the N and LD3 groups, indicating impaired vitamin D metabolism associated with estrogen deficiency (Figure 4). Administration of conventional vitamin D3 (D+) resulted in a non-significant change in serum levels (p>0.01, Brown-Forsythe and Games-Howell post-hoc), which indicated the limited bioavailability of the standard formulation compared to D– group. Notably, mice treated with LD3 demonstrated a greater elevation in serum vitamin D3 compared to the D+ group, although levels remained lower than in the non-surgical group. This finding underscored the enhanced systemic delivery and absorption conferred by the liposome formulation, and highlighted the potential of nanocarrier-based strategies to improve vitamin D3 bioavailability in the context of menopause-related deficiency.

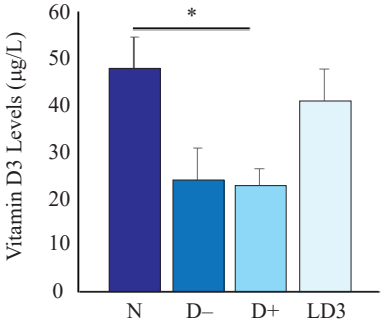

Figure 4. Serum vitamin D3 levels in experimental groups. Data are presented as mean±SEM (n=7 per group). Statistical significance was determined by Brown-Forsythe followed by Games-Howell post-hoc. *p<0.01.

Anti-inflammatory Effect of LD3 on IL-6 Levels

The ovariectomized group without vitamin D3 supplementation (D−) exhibited a significant increase in IL-6 levels (p<0.01, one-way ANOVA and Duncan's post-hoc test) compared with the non-surgical controls, confirming the inflammatory consequences of estrogen withdrawal (Figure 5). Both vitamin D3-supplemented groups, conventional (D+) and LD3, showed reductions in IL-6 relative to the D− group, demonstrating the anti-inflammatory effect of vitamin D3. However, these decreases were only partial, as IL-6 concentrations in both treatment groups remained higher than those in the non-surgical controls. Notably, the LD3 group showed levels comparable to the D+ group, suggesting only a modest advantage of the liposomal formulation in suppressing inflammatory responses. These findings supported the role of vitamin D3, particularly when delivered via nanocarriers, in modulating inflammation under estrogen-deficient conditions such as menopause.

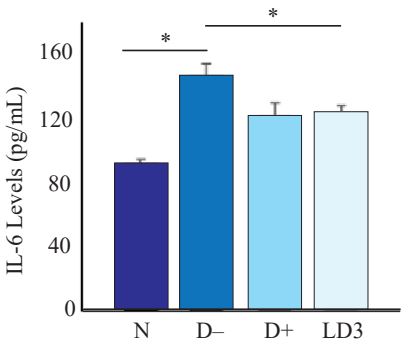

Figure 5. Serum IL-6 levels in experimental groups. Data are presented as mean±SEM (n=7 per group). Statistical significance was determined by one-way ANOVA followed by Duncan's post-hoc (*p<0.01).

Anti-inflammatory Effect of LD3 on TNF-α Levels

TNF-α levels in the untreated group were significantly increased (p<0.01, one-way ANOVA and Duncan's post-hoc test) compared with the non-surgical controls, indicating heightened systemic inflammation associated with estrogen deficiency (Figure 6). Supplementation with conventional vitamin D3 (D+) produced a moderate reduction in TNF-α levels, suggesting partial mitigation of the inflammatory response. Notably, the LD3 group exhibited a further and significant reduction in TNF-α compared with the D+ group (p<0.01, one-way ANOVA and Duncan's post-hoc test), with levels approaching those observed in the non-surgical group. This pattern highlights the superior efficacy of LD3 in suppressing proinflammatory cytokine expression, likely due to its enhanced bioavailability and cellular uptake via the nanocarrier system.

Vitamin D3 Levels Correlate Negatively with IL-6 and TNF-α Levels

A weak negative correlation was observed between the non-surgical group (N) and the D− and D+ groups, indicating that reduced vitamin D3 levels were associated with increased IL-6 concentrations under estrogen-deficient conditions. In contrast, a positive correlation was observed in the LD3-treated group, suggesting that liposomal supplementation was linked to a consistent reduction in inflammation. The negative correlation between the D− and LD groups, together with the positive correlation between the D+ and LD groups, implies a transitional pattern in which supplementation gradually restored the inflammatory profile toward normal levels (Figure 7A). These findings support the hypothesis that higher serum vitamin D3 levels are inversely associated with IL-6 and further suggest that LD3 was more effective in modulating this inflammatory biomarker due to its superior cellular uptake and systemic distribution

A negative correlation was observed between the non-surgical (N) and D− groups, reflecting the inflammatory shift induced by estrogen deficiency and the accompanying decrease in vitamin D3. Interestingly, a positive correlation was detected between the N and LD3 groups, suggesting that LD3 treatment in ovariectomized mice partially restored the immunological profile toward normal conditions. In contrast, the correlation between the D+ and LD3 groups was negative, indicating that conventional vitamin D3 supplementation was insufficient to suppress cytokine expression, likely due to its limited bioavailability (Figure 7B).

Discussion

The highest vitamin D3 levels were detected in the non-surgical group (N), reflecting expected physiological concentrations. In contrast, both the untreated vitamin D3-deficient group (D−) and the group treated with standard vitamin D3 (D+) exhibited lower vitamin D3 levels, indicating a persistent deficiency despite intervention in the latter (Figure 3). Interestingly, the group receiving LD3 demonstrated a significant increase in serum vitamin D3 levels compared with both the D− and D+ groups. This finding suggests that liposomal encapsulation enhances the bioavailability and absorption efficiency of vitamin D3, corroborating previous studies that highlight the therapeutic potential of nanoparticle-based delivery systems.(22) The liposomal formulation facilitates improved gastrointestinal uptake and protects the active compound from degradation,

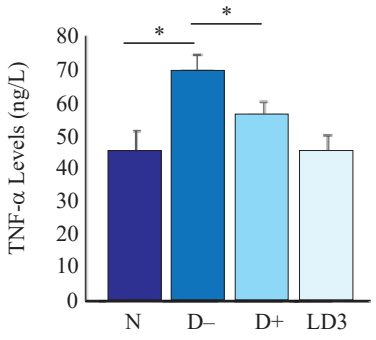

Figure 6. Serum TNF-α levels in experimental groups. Data are presented as mean±SEM (n=7 per group). Statistical significance was determined by one-way ANOVA followed by Duncan's post-hoc (*p<0.01).

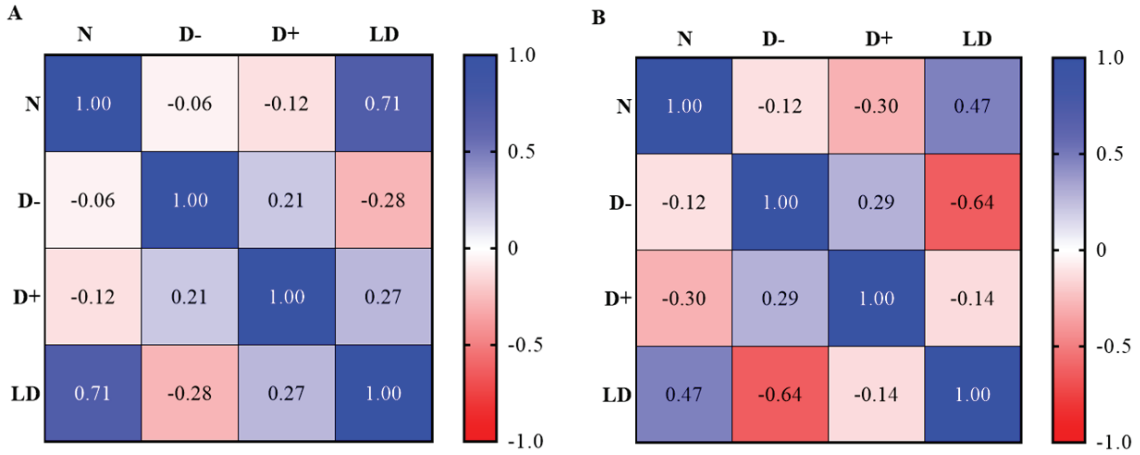

Figure 7. Correlation of vitamin D3 with IL-6 (A) and TNF-α (B). Dark blue represents a strong positive correlation (r → +1), dark red represents a strong negative correlation (r → −1), and white represents no or weak correlation (r ≈ 0). Matrix A (IL-6) seems to show moderate positive and negative correlations between the different groups. Matrix B (TNF-α) shows a more varied set of correlations, with some stronger negative correlations, especially between the LD3 group and others.

which may account for the superior outcomes observed in this group.

Previous clinical trials have consistently demonstrated the efficacy of standard vitamin D3 supplementation in elevating serum 25-hydroxyvitamin D [25(OH)D] concentrations in postmenopausal women.(23,24) For example, daily supplementation with 1000 IU of vitamin D3 over a 9-month period increased serum 25(OH)D levels by approximately 45.4%.(23) Nevertheless, the present study suggests that standard supplementation may not be sufficient for all individuals, particularly those undergoing menopause-related metabolic or absorptive alterations. These findings highlight the need for advanced delivery approaches, such as liposomal carriers, to optimize therapeutic outcomes

Furthermore, vitamin D3 sufficiency has been associated with a wide range of systemic benefits, including improved lipid profiles (24,25), a reduced risk of metabolic syndrome (23,26), enhanced bone mineral density and turnover (26-28), and a decreased severity of cardiovascular conditions such as coronary atherosclerosis (29). These pleiotropic effects highlight the essential role of vitamin D3 not only in musculoskeletal health but also in cardiometabolic regulation and systemic homeostasis. Consequently, strategies that enhance vitamin D3 bioavailability such as liposomal formulations may provide therapeutic advantages that extend well beyond the simple correction of deficiency. By improving the absorption efficiency and protecting vitamin D3 from premature degradation, liposomal delivery systems could help achieve more stable and sustained serum levels, thereby amplifying the broader clinical benefits of supplementation in populations vulnerable to estrogen deficiency related complications.

The findings of this study demonstrate that vitamin D3 supplementation, particularly in its liposomal formulation, exerts a pronounced anti-inflammatory effect by downregulating the expression of key proinflammatory cytokines, IL-6 and TNF-α. In the untreated disease group (D−), a significant elevation in both IL-6 and TNF-α levels was observed compared with the non-surgical group (N), indicating heightened immune activity consistent with ongoing inflammatory processes. These results are consistent with current scientific evidence highlighting IL-6 and TNF-α as central mediators in chronic inflammatory conditions, including periodontitis and systemic inflammatory disorders.(30,31) Taken together, these findings suggest that LD3 supplementation may hold translational relevance for the clinical management of inflammation-driven conditions beyond menopause. By effectively suppressing IL-6 and TNF-α, liposomal formulations not only address localized inflammatory responses but may also contribute to mitigating systemic complications linked to chronic low-grade inflammation. Such implications are particularly significant for postmenopausal women, who are predisposed to a heightened proinflammatory state due to estrogen deficiency. Consequently, the use of nanocarrier-based vitamin D3 delivery systems could represent a promising adjunct strategy for reducing the burden of systemic inflammatory disorders, with potential applications extending to metabolic syndrome, osteoporosis, and cardiovascular disease.(32,33)

The upregulation of these cytokines reflects the activation of inflammation-associated signaling cascades, such as the Toll-like receptor (TLR) 4/MyD88/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which orchestrates both innate and adaptive immune responses.(34) In contrast, treatment with conventional vitamin D3 and, more notably, with the liposomal formulation (LD3) resulted in a significant reduction in the expression of both cytokines. The LD3 group exhibited even lower levels of IL-6 and TNF-α compared with the D+ group, although these levels did not fully return to the baseline observed in healthy controls, particularly in the case of IL-6. Mechanistically, vitamin D3 has been shown to inhibit NF-κB activation by promoting the expression of inhibitor of NF-κB alpha (IκBα), a regulatory protein that sequesters NF-κB in the cytoplasm, thereby attenuating downstream transcription of proinflammatory mediators. Liposomal encapsulation further enhances this effect by improving cellular uptake and sustaining vitamin D3 bioavailability, which together may account for the greater anti-inflammatory efficacy observed in the LD3 group.

The superior efficacy of the liposomal formulation may be attributed to its enhanced bioavailability, improved tissue distribution, and more efficient cellular uptake compared with conventional vitamin D3. Previous studies have demonstrated that liposome-encapsulated vitamin D3 effectively targets immunocompetent cells such as dendritic cells and macrophages, modulating immune responses through the induction of regulatory T cells and the suppression of proinflammatory T helper type 1 (Th1) and Th17 pathways.(34,35) Furthermore, LD3 has been shown to inhibit toll-like receptor 4 (TLR4) signal transduction and downregulate the expression of adaptor proteins MyD88 and Toll/IL-1 receptor domain–containing adapter-inducing interferon-β (TRIF), thereby suppressing IL-6 and TNF-α production at the transcriptional level.(35)

Correlation analysis using a heat map further revealed a negative relationship between the D− and N groups and a strong positive correlation between the D+ and LD groups, reinforcing the hypothesis that vitamin D3 contributes to the normalization of cytokine expression under inflammatory conditions. These results are consistent with earlier reports showing that one-year supplementation with vitamin D3 reduced IL-6 and TNF-α levels and improved insulin sensitivity in obese individuals, thereby underscoring the immunomodulatory and metabolic regulatory roles of vitamin D3.(36) In addition, studies have demonstrated that vitamin D3 can significantly reduce the production of proinflammatory cytokines, particularly IL-6 and TNF-α, in patients with ulcerative colitis.(37)

Hence, these findings suggest that LD3 may represent a more effective therapeutic strategy for mitigating inflammatory responses than conventional formulations, particularly in the context of chronic diseases characterized by immune dysregulation. The advantages conferred by the liposomal delivery system open new avenues for the development of more targeted and efficient vitamin D3-based interventions, applicable to both systemic and localized therapies.(38,39) This study provides valuable insights into the molecular mechanisms underlying the immunoregulatory effects of vitamin D3 and underscores the potential of nanotechnology-enhanced formulations in advancing anti-inflammatory therapeutics.

Conclusion

Administration of vitamin D3-loaded liposomes effectively reduced the proinflammatory cytokines IL-6 and TNF-α, which are markedly elevated in response to estrogen deficiency. These findings underscore the potential of liposomal delivery systems to enhance the bioavailability and therapeutic efficacy of vitamin D3 in managing menopause-associated inflammatory responses. Moreover, the results support the use of nanocarrier-based interventions as a promising immunomodulatory strategy with broader applications in mitigating systemic complications related to estrogen deficiency.

Acknowledgments

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Authors Contribution

BWAK, SRD and AA were involved in conceptualizing and planning the research. Meanwhile, BWAK and SPAW conducted data acquisition/collection, calculated experimental data, performed analyses, drafted the manuscript and designed the figures, and interpreted the results. All authors contributed to the final critical revision of the manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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