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Dose-Dependent Effects of Maternal Vitamin D_3 on Offspring IL-6 and IL-1β in a Rat Model

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Abstract

Objective: Vitamin D_3 is increasingly recognized for its role in immune regulation, particularly in modulating proinflammatory cytokines during early development. This study aimed to investigate the dose-dependent effects of maternal vitamin D_3 supplementation during pregnancy and lactation on offspring inflammatory cytokine levels, focusing on interleukin-1 beta (IL-1β) and interleukin-6 (IL-6).

Materials and Methods: Twelve pregnant Sprague-Dawley rats were randomly assigned to four groups: one control and three treatment groups receiving vitamin D_3 at 62, 415 and 663 IU/kg body weight/day, respectively. Supplementation was administered orally from gestation day 1 to postnatal day 23. Offspring were subjected to an acute inflammatory challenge via lipopolysaccharide injection, after which induration size was measured. Serum levels of vitamin D_3, IL-1β and IL-6 in six offspring per group were quantified using enzyme-linked immunosorbent assay (ELISA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test.

Results: No significant difference was observed in post-injection induration size between groups ($p>0.05$). However, the group receiving 663 IU/kg/day vitamin D_3 exhibited significantly higher serum vitamin D_3 levels (21.15 ± 15.8 ng/mL) compared with controls (3.56 ± 3.20 ng/mL, $p=0.023$), along with significantly lower IL-1β level (33.5 ± 25.44 pg/mL, $p<0.001$). IL-6 levels showed a similar decreasing trend. Serum vitamin D_3 was moderately and inversely correlated with IL-1β ($r= -0.43$, $p=0.042$).

Conclusion: Maternal vitamin D_3 supplementation during gestation and lactation elevated serum vitamin D_3 and suppressed IL-1β and IL-6 levels in offspring, suggesting a dose-dependent immunomodulatory effect. These findings highlight the potential of maternal vitamin D_3 status to influence inflammatory responses during early life.

Keywords: Inflammation, IL-1β and IL-6, maternal supplementation, vitamin D_3

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Introduction

Vitamin D_3 deficiency is a significant health concern during pregnancy and lactation due to its potential long-term effects on both maternal and neonatal health (1,2). Vitamin D_3 supports calcium homeostasis, immune development, and inflammation regulation (3). Low maternal vitamin D_3 levels are associated with an increased risk of pregnancy complications, including preeclampsia and gestational diabetes, and studies have shown higher rates of neonatal asphyxia and immune dysregulation in infants born to vitamin D_3-deficient mothers (4). Boskabadi et al. (5) reported a higher prevalence of respiratory complications in premature infants of vitamin D_3-deficient mothers, highlighting the link between maternal vitamin D_3 status and neonatal outcomes (5). Furthermore, maternal deficiency reduces neonatal vitamin D_3 levels, increasing susceptibility to immune dysregulation and excessive inflammation (6,7).

During pregnancy and lactation, vitamin D_3 is transferred to the fetus and infant through the placenta and breast milk. It contributes to modulating immune responses by balancing cytokine production, thereby reducing the risk of allergies, chronic inflammation, and other immune-mediated conditions in early life (8). Studies have demonstrated that adequate maternal vitamin D_3 levels during pregnancy decrease the risk of chronic inflammation in infants and promote immune balance, thereby lowering the likelihood of allergic diseases and asthma (9,10). Notably, vitamin D_3 supplementation during pregnancy and lactation has been shown to promote immune homeostasis and reduce inflammation in offspring (9,11).

Vitamin D_3 deficiency has been associated with increased levels of pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and interleukin-1 beta (IL-1β), which are implicated in the development of inflammatory disorders in children (12). Although vitamin D_3 is widely recognized for its role in immune modulation, limited research has explored its dose-dependent impact during both pregnancy and lactation on offspring cytokine responses to acute inflammatory stimuli (13). To address this gap, the present study investigated the immunomodulatory effects of varying doses of maternal vitamin D_3 supplementation administered throughout gestation and lactation, with a focus on IL-6 and IL-1β expression in the offspring. A rat model is used to simulate maternal and offspring interactions and to support the development of early prevention strategies for inflammatory diseases.

No preclinical study has simultaneously implemented prenatal (gestational & lactational) vitamin D_3 supplementation, followed by acute lipopolysaccharide (LPS)-induced inflammation in offspring, with serum measurement of IL-6 and IL-1β at the 3-hour post-injection timepoint. Prior studies have focused on single markers (e.g., tumor necrosis factor alpha [TNF-α] without LPS challenge), organ-specific inflammation, or descriptive immune modulation without acute challenge. Our design fills this critical knowledge gap by targeting downstream cytokine responses in a time-sensitive, systemic inflammatory context.

Materials and Methods

Experimental Animals

Female founder (F0) *Rattus norvegicus* (Sprague-Dawley strain) rats were used as experimental animals. The animals were housed in a controlled environment at a temperature of 22 ± 2°C, with a relative humidity of 40–70% and a 12/12-hour light/dark cycle. They had unrestricted access to water and were provided with standard feed (30 grams/day) *ad libitum*. The study was conducted at the Experimental Animal Laboratory, Faculty of Medicine, Universitas Baiturrahmah, West Sumatra, Indonesia, in accordance with the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines. All experimental procedures were approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Baiturrahmah, West Sumatra, Indonesia (Approval No. 040/ETIK-FKUNBRAH/03/07/2024).

Study Design

This experimental *in vivo* study included 12 female Sprague Dawley rats (150–200 g, 8–12 weeks old). The schematic flow of the study is shown in Figure 1. All founder rats underwent a 14-day acclimatization phase before any intervention. After a 14-day acclimatization, founder rats (F0) were paired with males (1 male: 2 females per cage). Pregnancy was confirmed by the presence of a vaginal plug, marking gestational day 1, after which pregnant rats were housed individually (14).

Pregnant rats were randomly assigned to four groups. Three groups received standard feed diet combined with vitamin D_3 (Cat. No. 020734; SUPRA FERBINDO FARMA®, Jakarta, Indonesia) supplementation administered orally via gavage, with each group assigned to a specific supplementation dose (Supplement 1). Group A received

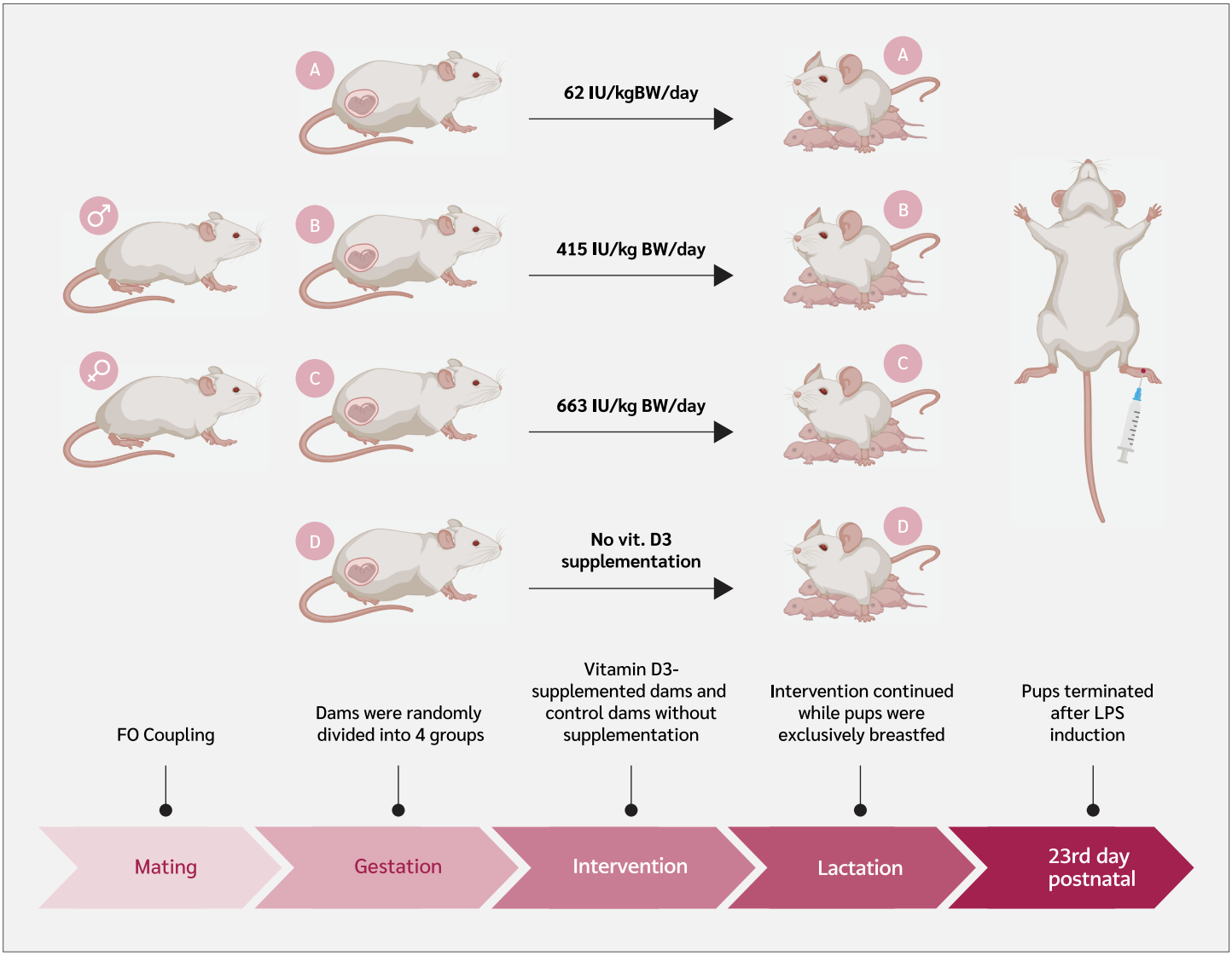

Figure 1. Vitamin D_3 supplementation and experimental intervention design (created with BioRender®, Canada; n=6 per group)

62 IU/kg body weight (BW)/day of vitamin D_3, Group B received 415 IU/kg BW/day of vitamin D_3, and Group C received 663 IU/kg BW/day of vitamin D_3. The vitamin D supplementation was provided separately from the standard diet to ensure accurate dosage administration. Group D served as the control and received no vitamin D_3 supplementation.

The supplementation doses were adjusted to experimental requirements using the Animal Equivalent Dose (AED) conversion formula based on the Km ratio. The human-to-rat dose conversion formula was:

AED (mg/kg) = Human dose (mg/kg) × Km ratio,

Where the Km value for rats is 6.2, and then the results are converted to IU/kg BW. Accordingly, 62 IU/kg BW/day in rats corresponds to 600 IU/day in humans, 415 IU/kg BW/day corresponds to 4000 IU/day, and 663 IU/kg BW/day corresponds to 6400 IU/day. These adjustments ensured the administered doses were physiologically relevant across species.

Rats received 30 grams of feed daily *ad libitum* and vitamin D_3 administered by oral gavage from gestational day 1 to postnatal day 23. Daily feed intake was measured, including spilled pellets collected from the sawdust. Before weaning on postnatal day 23, six offspring (according to Federer's formula) with similar body weights

per group were selected randomly, regardless of sex, for analysis of serum vitamin D_3 levels, acute inflammation response, edema, and serum IL-1β and IL-6 levels.

Inflammation Induction

On postnatal day 23, inflammation was induced in each offspring with a 100 µg/100 µL LPS (Cat. No. L2630-10MG; Sigma-Aldrich®, Darmstadt, Germany) injection into the paw (0.1 mL). The inflammatory response was assessed by measuring induration 3 hours post-injection, as described by Vajja (15), who found that pro-inflammatory cytokines, such as IL-6 and IL-1β, increased at 3 hours post-LPS injection.

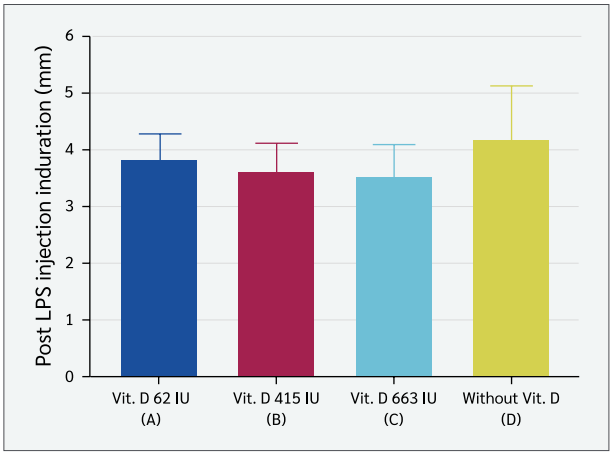

Figure 2. Mean induration diameter (mm) of offspring after lipopolysaccharide (LPS) induction (3 hours) (one-way ANOVA, p=0.37; n=6 per group).

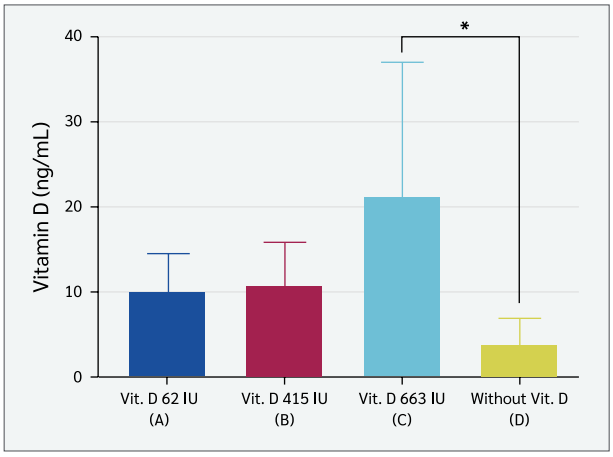

Figure 3. Serum vitamin D_3 levels (ng/mL) in offspring across the four treatment groups (one-way ANOVA, p=0.031).

*Data are presented as mean ± SD (n=6 per group). p=0.023 compared with the control group.

Sample Collection and Biochemical Analysis

Blood samples were collected from the left ventricle. Approximately 2 mL of blood was collected directly from the heart following euthanasia, which was performed using intraperitoneal administration of ketamine at a dose of 100 mg/kg BW. The collected blood was centrifuged at 5000 rpm for 10 minutes to separate the serum. The supernatant was transferred to a new tube and stored at -20°C. Serum vitamin D_3 concentrations were measured using the competitive enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. E-EL-0014; Elabscience®, USA). The detection limit for vitamin D_3 was 0.94 ng/mL, and the intra-assay coefficients were <10%. IL-1β and IL-6 concentrations were assessed using sandwich ELISA kits (Cat. No. E-EL-R0012; Elabscience®, Houston, TX, USA for IL-1β and Cat. No. E-EL-R0015; Elabscience®, Houston, TX, USA for IL-6). The intra-assay coefficients of variation for IL-1β were <10% with a sensitivity of 18.75 pg/mL, while for IL-6, the intra-assay coefficients of variation were <10% with a sensitivity of 7.5 pg/mL.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was used to assess data normality, and Levene's test was used to evaluate homogeneity of variances. For normally distributed data, two-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc testing was performed. For non-normally distributed data, the Kruskal-Wallis test followed by the Mann-Whitney post hoc test was applied. Correlations between variables were examined using Pearson's correlation coefficient. All results were expressed as mean ± standard deviation (SD), and statistical significance was set as p<0.05.

Results

Post-LPS Induction Induration Response

Inflammatory induction was performed by injecting LPS subcutaneously into the plantar surface of the offspring's paw, and induration was measured 3 hours post-injection. The induration measurements (mm) are presented in the graph below (Figure 2). No significant differences in induration diameter were observed among the groups following LPS injection (one-way ANOVA, p>0.05).

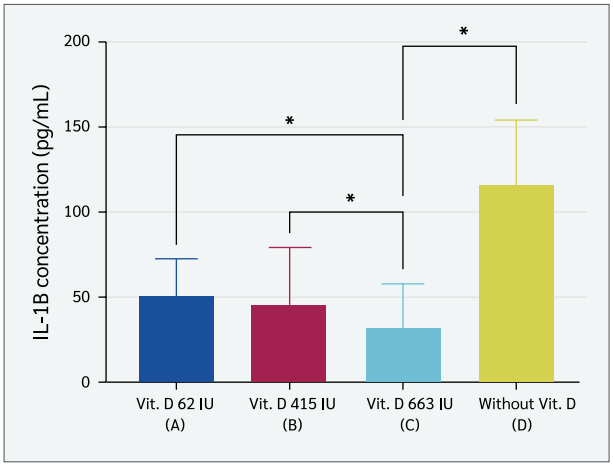

Figure 4. Serum interleukin-1β (IL-1β) levels (pg/mL) in the four offspring groups (one-way ANOVA, $p<0.001$; post hoc LSD test, $p=0.03$; $p=0.02$; $p<0.001$).

*Data are presented as mean ± SD (n=6 per group). $p\leq0.05$ compared with the control group.

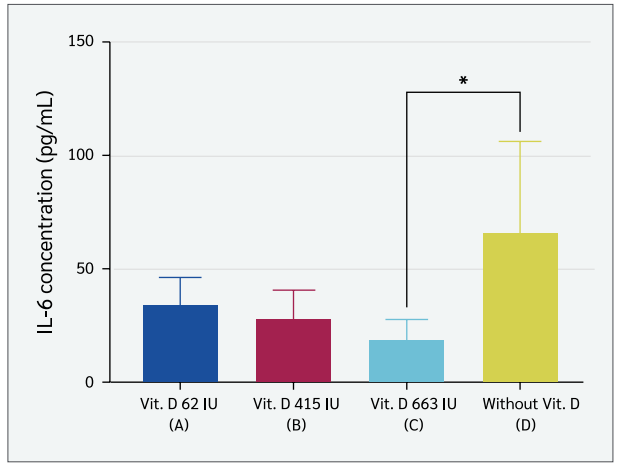

Figure 5. Serum interleukin-6 (IL-6) levels (pg/mL) in the four offspring groups (Kruskal-Wallis test, $p=0.037$).

*Data are presented as mean ± SD (n=6 per group). $p=0.023$ compared with the control group.

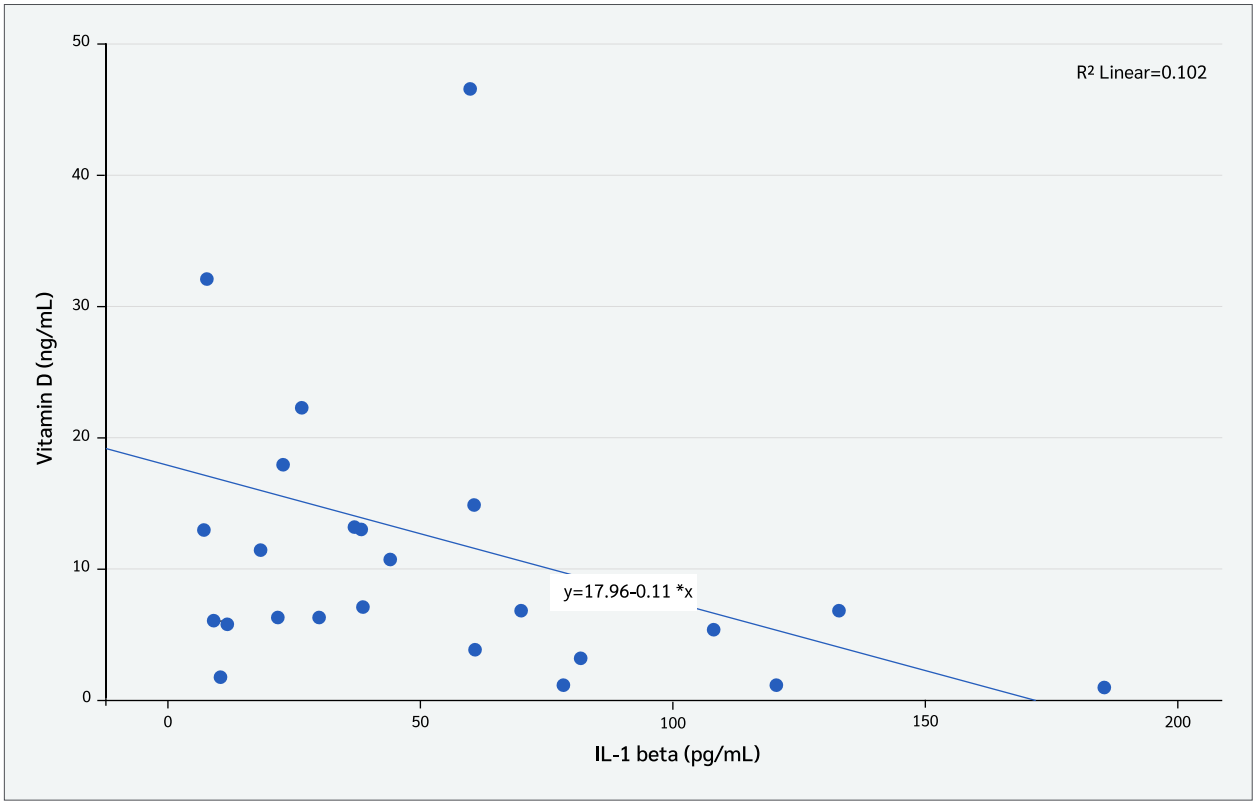

Figure 6. Correlation between serum vitamin D_3 levels (ng/mL) and serum interleukin-1β (IL-1β) concentrations (pg/mL) (Pearson's correlation, $r=-0.43$, $p=0.042$; n=24)

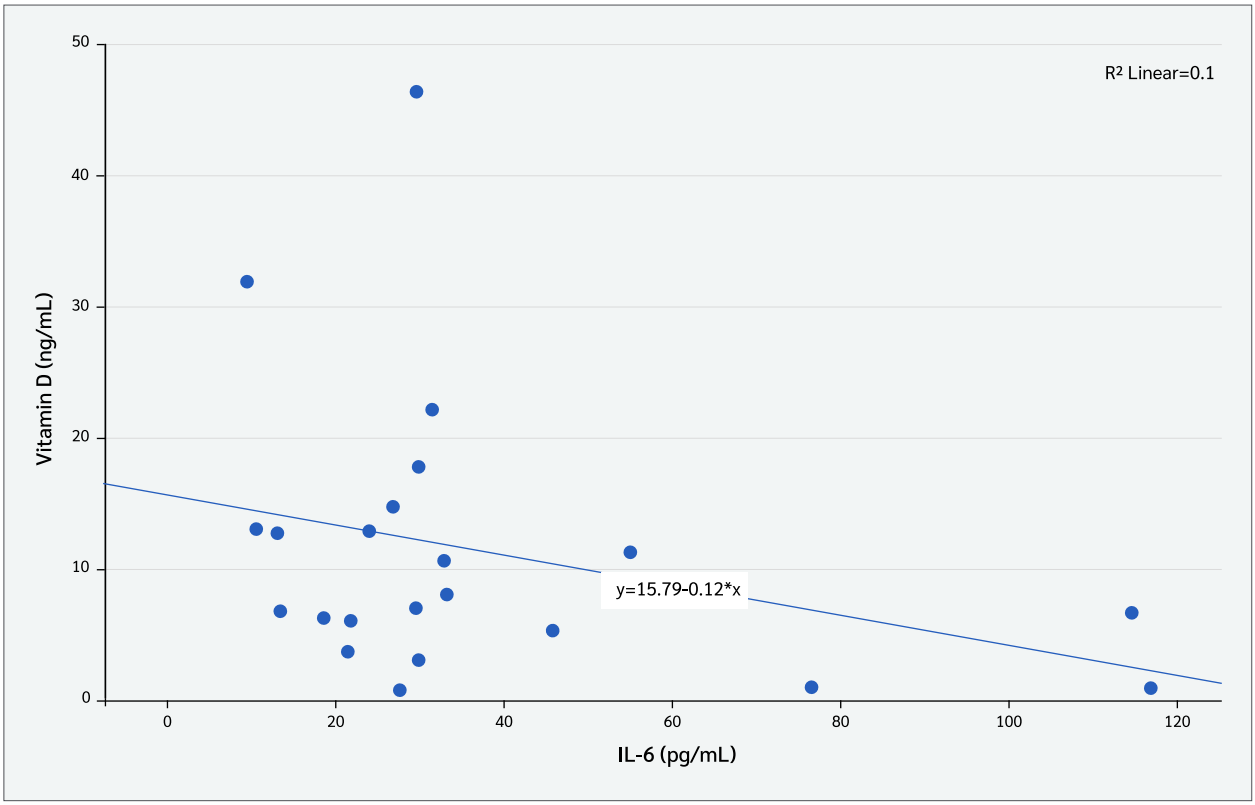

Figure 7. Correlation between serum vitamin D_3 levels (ng/mL) and serum interleukin-6 (IL-6) concentrations (pg/mL) (Pearson's correlation, r=-0.31, p=0.153; n=24).

Serum Vitamin D_3 Levels

Serum vitamin D_3 levels (ng/mL) in the four groups were assessed on day 23 after LPS induction. This study showed a significant difference in the mean levels (Figure 3) between the group of rats receiving 663 IU/kg BW of vitamin D_3 (21.15 ± 15.8 ng/mL) and the group that did not receive vitamin D_3 (3.56 ± 3.20 ng/mL) (one-way ANOVA, post hoc LSD, p=0.023).

Serum IL-1β level

Group C exhibited the lowest serum IL-1β levels, with a mean concentration of 33.5 ± 25.44 pg/mL (Figure 4). Statistical analysis (one-way ANOVA, p<0.001) showed IL-1β levels were significantly higher in low-dose vitamin D_3 groups (62 IU and 415 IU) than in the 663 IU group (LSD post hoc, p=0.03; p=0.02). The non-supplemented group also showed higher IL-1β levels than the 663 IU group (p<0.001), suggesting a modulatory effect of higher vitamin D_3 doses on inflammation.

Serum IL-6 Level

Serum IL-6 levels were lowest in Group C, with a mean of 19.64 ± 9.05 pg/mL (Figure 5). Kruskal-Wallis analysis showed a significant difference among groups (p=0.037), with post hoc LSD revealing lower IL-6 levels in Group C than the non-supplemented group (p=0.023).

Correlation Between Serum Vitamin D_3 Levels and Interleukin Levels

Correlation analyses were conducted to examine the relationship between serum vitamin D_3 levels (pg/mL) and interleukin levels. As shown in Figure 6, serum vitamin D_3 levels exhibited a moderate negative correlation with serum IL-1β concentrations (Pearson's correlation, r=-0.43, p=0.042). In contrast, no statistically significant correlation was observed between serum vitamin D_3 levels (pg/mL) and serum IL-6 concentrations (Pearson's correlation, r=-0.031, p=0.153) (Figure 7).

Discussion

The dosage selection in this study was based on previous research and established recommendations (16,17). A dose of 600 IU/day for humans, equivalent to 63 IU/kg BW in rats, was derived from the Institute of Medi-

cine (IOM) guidelines recommending 400–600 IU/day during pregnancy and lactation. However, studies have shown that this level is insufficient to maintain adequate vitamin D_3 status. The maximum dose of 6400 IU/day in humans (663 IU/kg BW in rats) was chosen because evidence indicates it meets maternal requirements during breastfeeding without adverse effects (16). An intermediate dose of 4000 IU/day (415 IU/kg BW in rats) was also included, as previous findings demonstrated its effectiveness in meeting vitamin D needs in lactating mothers (17).

This study investigated the effects of maternal vitamin D_3 supplementation on the offspring's inflammatory response to LPS, assessing induration diameter as a marker of local inflammation. The results showed no significant differences between groups, suggesting that maternal vitamin D_3 supplementation may not directly influence acute local inflammatory responses.

The physiological mechanism underlying LPS-induced induration involves TLR4-dependent activation of innate immune cells and release of pro-inflammatory cytokines—TNF-α, IL-1β, and IL-6—which drive endothelial activation, chemokine production, leukocyte infiltration, and localized swelling/induration (18,19). Although vitamin D_3 is known to downregulate these cytokines and enhance the production of anti-inflammatory cytokines such as IL-10 (20), its modulatory effects appear to be more prominent in chronic or systemic inflammation rather than in acute, localized immune responses (21). We selected IL-6 and IL-1β as primary pro-inflammatory readouts at 3 hours post-LPS. We did not assay TNF-α because its serum levels are known to peak much earlier (often within 1–2 hours post-stimulation) and rapidly decline, making detection at 3 hours susceptible to false negatives. In contrast, IL-6 and IL-1β have more sustained serum profiles and reliably reflect downstream inflammatory amplification (15,22).

Vitamin D_3 primarily modulates systemic inflammation, with studies linking high doses to reduced serum IL-1β and IL-6, but not localized inflammation (23). Additionally, the neonatal immune system is more tolerogenic. A study by Hughes and Norton (24) highlighted that neonatal immune responses tend to suppress excessive inflammation as a developmental adaptation, which may further limit induration diameter in offspring after maternal vitamin D_3 supplementation (25).

Maternal D_3 supplementation significantly altered the serum vitamin D_3 levels of offspring. Offspring from dams receiving 663 IU/kg BW exhibited markedly higher vitamin D_3 levels compared to the non-supplemented group (p=0.023), consistent with findings by Chien et al. (26), who reported that maternal intake exceeding 400 IU/day was associated with improved maternal and offspring outcomes. A clear dose-dependent response was observed, as the 663 IU/kg BW group showed substantially higher levels than controls (21.15 ± 15.8 ng/mL vs. 3.56 ± 3.20 ng/mL), whereas the 62 IU/kg BW group did not differ significantly from controls, suggesting that low doses are insufficient. This aligns with Hollis et al. (16), who noted that low-dose maternal supplementation fails to raise vitamin D_3 concentrations in breast milk, thereby increasing the risk of neonatal deficiency (27).

Maternal vitamin D_3 supplementation enhances the vitamin D_3 status of offspring through both placental transfer and breast milk, with higher maternal levels directly increasing neonatal vitamin D_3 concentrations (28,29). Hollis et al. (16) further emphasized the critical role of adequate maternal vitamin D_3 in optimal fetal development, underscoring the importance of supplementation during pregnancy (27). Additionally, the placental expression of vitamin D_3 metabolic enzymes, such as CYP24A1 and CYP27B1, is regulated, facilitating the transfer of vitamin D_3 to the fetus (30).

Postnatally, breast milk is a key source of vitamin D_3 for exclusively breastfed infants, and its vitamin D content is strongly dependent on maternal intake; high-dose maternal supplementation (≈6000–6400 IU/day) increases milk vitamin D_3 and achieves infant vitamin D sufficiency comparable to direct infant supplementation, thereby lowering the risk of neonatal deficiency (16,31). Adequate maternal supplementation increases milk vitamin D_3 levels, thereby reducing the risk of neonatal deficiency (32,33). Conversely, exclusive breastfeeding without sufficient maternal vitamin D_3 may result in infant deficiency (33). Clinical studies further emphasize the importance of maternal supplementation in maintaining adequate vitamin D_3 levels in infants (34).

Maternal vitamin D_3 supplementation significantly attenuated offspring inflammatory responses, reflected by lower serum IL-1β and IL-6 levels following LPS-induced inflammation. The highest dose of supplementation (663 IU/kg) produced the most pronounced effect, yielding the lowest IL-1β concentration and indicating

a dose-dependent anti-inflammatory effect ($p<0.001$). In contrast, offspring from dams receiving lower doses (62 IU and 415 IU) and the non-supplemented displayed markedly higher IL-1β levels. These findings support the notion that higher maternal vitamin D_3 intake more effectively mitigates inflammation and are consistent with a previous report describing the immunomodulatory capacity of vitamin D_3 in downregulating pro-inflammatory cytokines and limiting excessive immune activation (35).

IL-1β is a pivotal mediator of systemic inflammation, produced predominantly by monocytes and macrophages in response to pathogens or endotoxins (36). It amplifies inflammatory signaling by upregulating inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (23,37), while simultaneously promoting the release of IL-6 and TNF-α, thereby sustaining inflammatory cascades and contributing to chronic inflammatory diseases (38-41). In this study, offspring from dams supplemented with 663 IU/kg vitamin D_3 exhibited the lowest IL-1β levels which were significantly lower than those of the non-supplemented group. These results indicate that maternal vitamin D_3 supplementation at higher doses is capable of suppressing acute inflammatory responses by downregulating IL-1β production. Mechanistically, vitamin D_3 exerts these effects by inhibiting NLRP3 inflammasome activation, reducing caspase-1–mediated IL-1β maturation, and promoting macrophage polarization toward the M2 phenotype, which enhances IL-10 secretion while suppressing pro-inflammatory cytokines (42-45). Furthermore, vitamin D_3 downregulates Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-κB) signaling, key pathways in LPS-induced IL-1β expression, and restrains Th17 differentiation, thereby controlling IL-17–driven inflammation (46-48).

In addition to IL-1β, maternal vitamin D_3 supplementation also reduced serum IL-6 concentrations in offspring. The lowest IL-6 level was observed in the 663 IU/kg group, which was significantly different from the non-supplemented group. IL-6 is a pleiotropic cytokine secreted by macrophages, dendritic cells, T lymphocytes, and fibroblasts in response to infection or tissue injury (49). It regulates immune cell activation, antibody production, and acute-phase responses, and persistent elevation is linked to chronic inflammatory conditions such as type 2 diabetes, autoimmune diseases, and atherosclerosis (37,40,41). Lipopolysaccharide, the main component of Gram negative bacterial cell walls, stimulates IL-6 production by binding TLR4 and activating NF-κB signaling, which also drives the release of IL-1β and TNF-α (38-41). By suppressing NF-κB activation and preventing its nuclear translocation, vitamin D_3 downregulates IL-6 expression (39,49). Vitamin D_3 also reprograms macrophages from a pro-inflammatory M1 state to an anti-inflammatory M2 state, reduces IL-6 while increasing IL-10 secretion, and inhibits Th17 cells that normally amplify IL-6 production, while supporting regulatory T cells that maintain immune tolerance (23, 50-52). These findings are in line with research by Gatera et al. (52), which reported that vitamin D_3 reduces LPS-induced IL-6 synthesis by interfering with NF-κB signaling. Collectively, these mechanisms demonstrate the capacity of vitamin D_3 to modulate LPS-triggered inflammation through coordinated suppression of both IL-1β and IL-6.

Correlation analyses further confirmed the anti-inflammatory role of vitamin D_3. A moderate negative correlation was observed between serum vitamin D_3 and IL-1β levels, whereas no significant relationship was detected with IL-6. These findings aligned with earlier studies demonstrating that higher vitamin D_3 status was associated with lower IL-1β levels (54,55), and may indicate that IL-1β is a more sensitive biomarker of immunomodulatory effects of vitamin D_3 in acute inflammation. Additional evidence suggests that vitamin D_3 supplementation suppresses both IL-1β and IL-6 by modulating mitogen-activated protein kinase (MAPK) phosphatase-1, thereby alleviating LPS-induced inflammatory responses (39). Taken together, these results provide compelling evidence that maternal vitamin D_3 supplementation not only reduces pro-inflammatory cytokine production in offspring but also enhances immune regulation, supporting its potential role as a preventive strategy against excessive inflammatory activation in early life (56,57).

Conclusion

Maternal vitamin D_3 supplementation during pregnancy and lactation increased offspring serum vitamin D_3 levels and reduced IL-6 and IL-1β concentrations, demonstrating a dose-dependent role in regulating systemic inflammation. The negative correlation between vitamin D_3 and IL-1β highlights its potential as an immunomodulatory agent, promoting immune homeostasis in the offspring.

Ethical Approval: The study was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Baiturrahmah, on July 3, 2024, with decision number 040/ETIK-FKUNBRAH/03/07/2024.

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

Author Contributions: Concept – K.M.H., W.S., G.K., A.D., L.Z., A.L.M.; Design – K.M.H., W.S., A.D., R.M., M.S.; Supervision – K.M.H., W.S., A.D., R.M.; Fundings – K.M.H., W.S.; Materials – K.M.H., W.S., G.K., A.D., M.S., R.M.; Data Collection and/or Processing – K.M.H., W.S., GF, A.D., M.S.; Analysis and/or Interpretation – K.M.H., W.S., G.K., A.L.M.; Literature Review – K.M.H., W.S., G.K., L.Z.; Writer – K.M.H., W.S., G.K., A.D., R.M., M.S., L.Z., A.L.M.; Critical Reviews – K.M.H., W.S., G.K., A.D., R.M., M.S., L.Z., A.L.M.

Conflict of Interest: The authors declare no conflict of interest.

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