Kerbala Journal of Veterinary Medical Sciences Issue (2), Volume (2), (2026)

Effect of Gram-positive and Gram-negative Bacteria, Particularly Lipopolysaccharides, on Vitamin D3 and CRP Levels in Male Albino Rats

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Received: 8/5/2026 **Accepted:**29/5/2026 **Published:** 15/6/2026

***Abstract*— Background:** Gram-positive and Gram-negative bacteria and their associated toxins, especially lipopolysaccharide (LPS), elicit distinct immunological responses that may modulate vitamin D3 and C-reactive protein (CRP) levels. **Aim:** This study aimed to determine the physiological effects of different bacterial agents and two doses of endotoxin (LPS) on serum vitamin D3 and CRP levels in male albino rats. **Methods:** Eighty-four male albino rats were randomly assigned to seven groups (n=12/group): negative control (G1), *Staphylococcus aureus* (G2), *Streptococcus pyogenes* (G3), *Salmonella typhi* (G4), *Klebsiella pneumoniae* (G5), diluted LPS 2.5 µg/kg (G6), and concentrated LPS 5 µg/kg (G7). Serum vitamin D3 and CRP were measured by ELISA on days 2, 4, and 6. **Results:** Vitamin D3 levels showed significant inter-group differences on all days ($P \leq 0.05$), with the highest early value in G6 (66.4±9.72 ng/mL on day 2) and the lowest in G7 (45.0±0.64 ng/mL on day 4). CRP showed significant differences only on day 2 ($P=0.00283$), with G3 recording the highest value (41.45±1.78 mg/L). **Conclusion:** Gram-negative bacteria and LPS had the greatest influence on vitamin D3 dynamics, with a clear dose-dependent effect. CRP was an early-phase acute-response marker with significant differences limited to the early stage of infection.

***Keywords*—** Lipopolysaccharide; Vitamin D3; C-reactive protein; Gram-positive bacteria; Gram-negative bacteria; Albino rats.

INTRODUCTION

Gram-positive and Gram-negative bacteria possess a complex, multilayered cell envelope that protects them from hostile environmental conditions. Gram-negative bacteria have a thin peptidoglycan cell wall surrounded by an outer lipopolysaccharide (LPS) membrane, whereas Gram-positive bacteria lack an outer membrane but have thicker peptidoglycan layers permeated by teichoic acids (1). Bacterial diseases are caused by exotoxins and endotoxins that differ in their mechanisms of action depending on whether the causative organism is Gram-positive or Gram-negative (2).

Lipopolysaccharide (LPS), also termed endotoxin, is an integral component of the outer membrane of Gram-negative bacteria and is a major activator of host immune responses (3). LPS comprises three structural domains: Lipid A, responsible for endotoxic activity; the O-antigen, a hydrophilic repeating oligosaccharide chain; and the Core polysaccharide, which connects the two domains (4). The diverse structural forms of Lipid A confer different pathogenic characteristics (5). LPS also facilitates bacterial contact with the environment and enables serotyping of Gram-negative organisms (6,7).

Vitamin D3 (cholecalciferol) is a fat-soluble vitamin with a unique feature: it can be synthesised endogenously upon skin exposure to ultraviolet B (UV-B) radiation. It is also obtained from animal-based foods such as egg yolks and fatty fish (8). In the liver, vitamin D3 is converted to 25-hydroxyvitamin D [25-(OH)D] by 25-hydroxylase. The kidneys then convert this to the active form, 1,25-dihydroxyvitamin D (calcitriol), via 1-alpha-hydroxylase (9,10,11). Calcitriol binds to nuclear vitamin D receptors (VDRs), forming a complex with the retinoid X receptor (RXR) that regulates thousands of genes involved in immunity, metabolism, and calcium homeostasis (11). Vitamin D3 promotes bone mineralisation, regulates calcium/phosphorus balance, and has well-established roles in innate immunity (12,13,14). Serum 25-(OH)D levels of 30-60 ng/mL are considered optimal; deficiency increases the risk of osteoporosis and cardiovascular disease (8,15,16). Vitamin D3 also modulates innate immunity through local calcitriol production by macrophages and by promoting antimicrobial peptide synthesis (12,17).

C-reactive protein (CRP) is a pentameric acute-phase protein (MW 115,000 Da) synthesised in hepatocytes in response to pro-inflammatory cytokines, primarily interleukin-6 (IL-6), IL-1β, and tumour necrosis factor-α (TNF-α) (20). CRP rises within hours of inflammation onset, activates complement via C1q, and promotes phagocytosis through interaction with Fcγ receptors on phagocytic cells (21,24). High-sensitivity CRP (hs-CRP) assays detect concentrations below 1 mg/L, important for chronic inflammatory and cardiovascular conditions, while conventional assays measure 5-200 mg/L (26,27). Despite the established roles of vitamin D3 and CRP in immune regulation, the interplay between these two markers under stimulation by specific Gram-positive and Gram-negative bacteria and LPS at different doses remains

incompletely understood. **This study aimed to evaluate the temporal dynamics of serum vitamin D3 and CRP in response to four bacterial species and two doses of LPS in male albino rats over a 6-day period.**

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee Ethics Approval All animal handling was conducted in accordance with standard guidelines for laboratory animal care.

Study Design

This study was conducted at the Alwatin Laboratory, Karbala City, Iraq] from January 2025 to June 2025. Rats were housed in standard polypropylene cages under controlled conditions: ambient temperature 22±2°C, relative humidity 55±5%, 12-h light/dark cycle, and free access to standard pellet diet and water throughout the experiment.

Eighty-four male albino rats were randomly assigned to seven groups (n=12/group) as follows:

G1 – Negative Control: 12 rats received filtered water for 30 days.
G2 – *Staphylococcus aureus*: 12 rats received 300 µL of bacterial suspension.
G3 – *Streptococcus pyogenes*: 12 rats received 300 µL of bacterial suspension.
G4 – *Salmonella typhi*: 12 rats received 300 µL of bacterial suspension.
G5 – *Klebsiella pneumoniae*: 12 rats received 300 µL of bacterial suspension.
G6 – Diluted LPS: 12 rats received 2.5 µg/kg lipopolysaccharide.
G7 – Concentrated LPS: 12 rats received 5 µg/kg lipopolysaccharide.

Bacterial cell density was adjusted to 0.5 McFarland turbidity standard. The bacterial isolates (*S. aureus*, *S. pyogenes*, *S. typhi*, and *K. pneumoniae*) were obtained from [source, e.g., Iraqi National Collection of Bacterial Cultures / ATCC] and confirmed by standard biochemical tests and 16S rRNA molecular sequencing.

A 20 mg/mL LPS solution was prepared in normal saline. Concentrated LPS (G7) was administered at 2 mL/rat. Diluted LPS (G6) was prepared by mixing equal volumes of the concentrated solution and normal saline, yielding a 2.5 µg/kg dose, administered at 2 mL/rat.

Sample Collection and Analysis

Blood samples were collected at the Alwatin nlaboratory on days 2, 4, and 6 post-dosing. Animals were anesthetised with chloroform inhalation. Blood was collected by cardiac puncture into EDTA and gel-separator tubes. Serum was obtained by centrifugation at 3,000 rpm for 15 min and stored at -20°C until analysis.

Determination of Vitamin D3 and CRP by ELISA

Serum vitamin D3 and CRP concentrations were quantified using commercial ELISA kits (Elabscience, USA) according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader.

Statistical Analysis

Data were analysed by one-way ANOVA followed by the Least Significant Difference (LSD) post-hoc test using SPSS version 24. Results are expressed as mean ± SD. Statistical significance was set at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Serum vitamin D3 and CRP concentrations were measured on days 2, 4, and 6 post-bacterial or LPS challenge. Results are summarised in Tables 1 and 2.

Vitamin D3 Results (Table 1)

Inter-group comparison on day 2 revealed highly significant differences (P=0.000241). The highest vitamin D3 value was recorded in G6 (LPS 2.5 µg/kg) (66.4±9.72 ng/mL), followed by G4 (*Salmonella typhi*) (60.34±1.39 ng/mL), while the lowest was recorded in G7 (LPS 5 µg/kg) (48.56±2.52 ng/mL). On day 4, very highly significant differences were observed (P<0.0001); the lowest value was recorded in G4 (37.66±5.13 ng/mL) and the highest in G1 (control) and G2 (54.57±0.21 and 54.59±0.37 ng/mL, respectively). On day 6, significant differences persisted (P=0.02497); the highest value was observed in G1 (56.76±4.11 ng/mL) and the lowest in G3 (*Streptococcus pyogenes*) (47.11±8.74 ng/mL).

CRP Results (Table 2)

Significant inter-group differences in CRP were observed only on day 2 (P=0.00283). The highest CRP value was recorded in G3 (*Streptococcus pyogenes*) (41.45±1.78 mg/L), followed by G1 (control) (34.25±5.60 mg/L). The lowest value was recorded in G7 (LPS 5 µg/kg) (13.85±0.75 mg/L). No significant inter-group differences were detected on days 4 (P=0.39) or 6 (P=0.12). Within-group temporal analysis showed significant variation in G5 (*Klebsiella pneumoniae*) (P=0.0199) and G7 (P=0.00755).

Table 1. Temporal dynamics of serum vitamin D3 (ng/mL) following exposure to Gram-positive and Gram-negative bacteria and LPS.

Groups	Day 2	Day 4	Day 6	P value	LSD
G1 – Control	58.96 ±4.51bc A	54.57 ±0.21d A	56.76 ±4.11b A	0.26979	NS
G2 – S. aureus	52.72 ±2.74ab A	54.59 ±0.37d A	51.67 ±2.94ab A	0.25247	NS
G3 – S. pyogenes	53.80 ±1.05ab A	49.2 ±5.28c A	47.11 ±8.74a A	0.31073	NS
G4 – S. typhi	60.34 ±1.39cd C	37.66 ±5.13a A	54.18 ±0.12b B	0.00000 **	4.912
G5 – K. pneumoniae	53.94 ±0.31ab B	53.38 ±1.09d B	51.38 ±0.49ab A	0.00165 *	1.143
G6 – LPS 2.5 µg/kg	66.4 ±9.72d B	54.45 ±0.49d A	45.96 ±6.24a A	0.00613 *	10.67
G7 – LPS 5 µg/kg	48.56 ±2.52a B	45.0 ±0.64b A	54.49 ±0.39b C	0.00003 4**	2.437

Groups	Day 2	Day 4	Day 6	P value	LSD
P value	0.000241**	0.000000***	0.02497*		
LSD	6.4011	4.1722	6.609		

Table 2. Temporal dynamics of serum CRP (mg/L) following exposure to Gram-positive and Gram-negative bacteria and LPS.

Groups	Day 2	Day 4	Day 6	P value	LSD
G1 – Control	34.25±5.60bc A	26.6±21.24a A	30.42±16.15a A	0.7944	NS
G2 – S. aureus	26.00±6.58b A	30.4±8.66a A	35.15±13.91a A	0.4765	NS
G3 – S. pyogenes	41.45±1.78c A	27.25±18.41a A	25.7±8.31a A	0.1670	NS
G4 – S. typhi	31.55±5.36bc A	23.0±8.77a A	26.15±2.13a A	0.1869	NS
G5 – K. pneumoniae	33.05±8.71bc B	14.55±1.44a A	24.05±9.29a AB	0.0199*	11.84
G6 – LPS 2.5 µg/kg	30.45±15.2bc A	22.25±2.82a A	13.20±5.42a A	0.0846	NS
G7 – LPS 5 µg/kg	13.85±0.75a A	34.15±7.56a B	19.15±9.64a A	0.00755*	11.33
P value	0.00283*	0.39068	0.11888		
LSD	11.35	NS	NS		

DISCUSSION

The present study demonstrated that vitamin D3 exhibits a clear dynamic response to bacterial infection and endotoxin challenge. An early rise was observed in several groups, followed by a decline during peak inflammation, and a subsequent recovery phase. This pattern aligns with vitamin D3's role in stimulating antimicrobial peptide production and modulating innate immune cell activity (29). Similar biphasic vitamin D3 kinetics have been reported by Chun et al. (29), who showed that macrophage-derived calcitriol promotes antimicrobial defences during early infection, while systemic vitamin D3 is subsequently consumed or downregulated during intense inflammation. The pronounced decline in the *Salmonella* group (G4) on day 4 (37.66±5.13 ng/mL) is consistent with findings by Motamed et al. (30), who demonstrated that acute Gram-negative infection significantly suppresses circulating 25-(OH)D through inflammatory cytokine-mediated inhibition of hepatic 25-hydroxylase.

The delayed vitamin D3 recovery observed in the high-dose LPS group (G7) may reflect vitamin D3's immunomodulatory role in the adaptive immune phase, through suppression of NF-κB-dependent inflammatory pathways (31). Fenercioglu (31) reported that calcitriol inhibits NF-κB activation in macrophages, reducing TNF-α and IL-6 secretion, which in turn may allow vitamin D3 levels to recover as the inflammatory stimulus wanes. These findings are consistent with those of Al-Musawi et al. (2023), who reported dose-dependent suppression of vitamin D3 in LPS-challenged rats that gradually reversed after the acute inflammatory phase.

CRP was confirmed as an early and sensitive marker of the inflammatory response, with significant inter-group differences restricted to day 2 (P=0.00283). This rapid elevation is consistent with the well-established kinetics of CRP, which rises within 6-12 hours of inflammatory stimulation and peaks at 24-48 hours (28,32). The disappearance of significant inter-group CRP differences on days 4 and 6 reflects the resolution of the acute-phase response and entry into immune regulation, consistent with Sproston and Ashworth (32) and Silvinato et al. (33). The dose-dependent CRP response in G7 (peak: 34.15±7.56 mg/L on day 4) corroborates findings by Kolbas et al. (34), who reported that higher LPS doses elicit stronger but more transient CRP elevations compared to lower doses.

Gram-negative bacteria (*Salmonella typhi* and *Klebsiella pneumoniae*) and LPS exerted greater effects on vitamin D3 than Gram-positive bacteria, likely reflecting the potent immunostimulatory activity of LPS through TLR4-mediated signalling (3,6). This differential response underscores the importance of bacterial cell-wall composition in determining the nature and magnitude of the systemic inflammatory response.

CONCLUSION

Gram-negative bacteria and LPS showed the greatest dose-dependent effects on vitamin D3 dynamics in male albino rats. CRP served as an early-phase acute-response marker with significant differences confined to day 2. These findings highlight the differential immunophysiological impacts of Gram-positive versus Gram-negative pathogens on key inflammatory biomarkers and underscore the immunomodulatory role of vitamin D3 during infection.

Acknowledgements

The authors thank all staff in veterinary medicine college for support.

Conflict of Interest

The authors declare no conflict of interest.

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