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High-dose Vitamin D Supplementation Attenuates NLRP3 Inflammasome-mediated Oxidative Stress in a Novel Murine Model of Comorbid Asthma and Osteoporosis Induced by Vitamin D Deficiency

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ABSTRACT

While vitamin D deficiency (VDD) is implicated in both asthma and osteoporosis, the synergistic mechanisms linking these comorbidities remain unexplored. This study introduces a novel murine model of VDD-induced concurrent asthma and osteoporosis, uniquely addressing their bidirectional exacerbation through NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and oxidative stress crosstalk.

Female C57 mice were stratified into control, bronchial asthma (BA), osteoporosis (OP), BA+OP, and VDD+BA+OP groups, with therapeutic evaluation of low-dose (LD) and high-dose (HD) vitamin D supplementation.

Unlike prior studies, our results demonstrate that VDD amplifies airway resistance and bone microstructural deterioration via NLRP3-driven pyroptosis (elevated cleaved caspase-1, N-terminal gasdermin D and suppressed antioxidant defenses (reduced glutathione peroxidase and catalase, and elevated malondialdehyde). Critically, HD supplementation reversed these effects more robustly than LD, restoring pulmonary compliance, trabecular integrity (bone volume/total volume: 0.0298 vs 0.0356 in VDD+BA+OP), and suppressing inflammasome activity. Mechanistically, we identify a feedforward loop wherein VDD-induced oxidative stress primes NLRP3 activation, which further exacerbates inflammation and bone resorption—a pathway uniquely mitigated by HD vitamin D.

These findings provide the first evidence of HD vitamin D's dual therapeutic efficacy in

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comorbid asthma-osteoporosis, offering a paradigm shift in targeting the NLRP3/oxidative stress axis for managing multifactorial inflammatory diseases.

Keywords: High-dose supplementation; Comorbid asthma-osteoporosis; NLRP3 inflammasome; Oxidative stress; Vitamin D deficiency

INTRODUCTION

Bronchial asthma is a complex disease marked by chronic airway inflammation, with symptoms that progressively worsen over time.[1] The incidence of asthma is rising annually, significantly impacting patients' quality of life and placing substantial burdens on families and society.[2] A positive link has been observed between vitamin D levels and asthma severity, the frequency of exacerbations, and disease worsening.[3-4] An epidemiological study from the United States found that the prevalence of asthma decreased along with a reduction in vitamin D deficiency (VDD).[5] Furthermore, vitamin D supplementation reduced the relative risk of asthma flare-ups by 26%.[6] Besides its role in asthma, vitamin D is a fat-soluble nutrient essential for calcium and phosphorus metabolism, playing a vital role in maintaining bone health. VDD leads to an increased turnover of bones and accelerates bone resorption, contributing to osteoporosis. These findings underscore the importance of vitamin D in managing comorbidities such as asthma and osteoporosis.

Osteoporosis (OP) is a common skeletal disorder marked by decreased bone mass and the degradation of bone tissue, leading to increased fragility and a higher risk of fractures.[7] Osteoporotic fractures are a major cause of disability and mortality among elderly populations, with substantial treatment expenses. Among the modifiable risk factors for osteoporosis, VDD is particularly important.[8,9] In the interplay between bronchial asthma and osteoporosis, VDD is a critical factor that exacerbates both conditions, intensifying their progression. Bronchial asthma, a chronic inflammatory lung disease, becomes more severe as systemic inflammatory cytokines increase. This escalation promotes osteoclast activity and directly disrupts bone metabolism, contributing to osteoporosis.

Therefore, this study aims to establish a mouse model of VDD-induced asthma combined with osteoporosis, systematically exploring the roles of oxidative stress and NLR family pyrin domain containing 3 (NLRP3) inflammasome expression in the underlying mechanisms.

MATERIALS AND METHODS

Mouse Model Establishment

Twenty-five female C57 mice, aged 6–8 weeks, were acclimatized and randomly assigned to five groups: control, OP, asthma (BA), BA+OP, and VDD-induced asthma with osteoporosis (VDD+BA+OP). Except for the control group, the osteoporosis model was established by ovariectomy in the other groups. To induce VDD, mice in the VDD+BA+OP group were fed a specially formulated vitamin D-deficient diet throughout the study. Control and OP groups received 200 μL phosphate-buffered saline (PBS) intraperitoneally on days 0, 7, and 21, followed by 50 μL PBS intranasally from days 28 to 30. BA, OP+BA, and VDD+BA+OP groups were sensitized with intraperitoneal injections of 200 μL PBS containing 50 μg ovalbumin (OVA) and 16 μg alum hydroxide on days 0, 7, and 21, followed by 50 μL 1 mg/mL OVA intranasally from days 28 to 30. Mice in the VDD+BA+OP group were fed a vitamin D-deficient diet throughout the study. After the 30-day experiment, asthma-related symptoms were assessed. Groups were further divided into control, VDD+BA+OP, low-dose (LD), and high-dose (HD).

Evaluation of Asthma-Related Symptoms

Observations were made on days 25, 26, and 27 during nebulization stimulation. The time of the first occurrence of each symptom was recorded as T1 (time to cyanosis), T2 (time to frequent scratching of the mouth/nose/limbs), and T3 (time to hunched posture with erect stance).

Assessment of Airway Hyperresponsiveness

Lung function was measured using the Ani Res2005 pulmonary analysis system.

HE Staining

The tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (HE) (Sigma, USA) for observation under a light microscope (OLYMPUS, Japan).

Immunohistochemistry

Lung and bone tissue sections underwent fixation, dehydration, clearing, embedding, sectioning, and hydration. Sections were blocked with goat serum for 20 minutes and incubated with primary antibodies (anti-Cyp2R1, 1:100; anti-Cyp24a1, 1:100; anti-vitamin D receptor (VDR), bs-10618R, 1:100, Bioss, China) for 1 hour. After secondary antibody incubation, slides were mounted for observation.

Biomechanical Testing

The right femur from each group, after removing soft tissues, skin, and muscles, was subjected to a three-point bending test using the ELF 3220 biomechanical testing system.

μCT Scanning

The left femurs were fixed in 4% paraformaldehyde for 72 hours and scanned using a micro-CT system (GE Healthcare, Canada).

Western Blot

The total protein was extracted and cleaved with radioimmunoprecipitation assay buffer (RIPA) (SolabioChina). After quantification, the proteins were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the membrane according to the steps. Membranes were blocked for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies (caspase11, interleukin [IL]-1β, IL-18, apoptosis-associated speck-like protein containing a CARD [ASC], NLRP3, Cleaved Caspase1, glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). After washing, membranes were treated with secondary antibodies (1:5000), washed with Tris-buffered saline with Tween 20, and visualized using an enhanced chemiluminescence (ECL) detection kit (Bio-Rad, USA).

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed according to the instructions provided with the ELISA kits.

Statistical Analysis

Data analysis was performed using Prism 6.0. Group differences were assessed with one-way analysis of variance (ANOVA), and statistical significance was set at $p<0.05$.

RESULTS

VDD Exacerbates Asthma Symptoms

Body weight increased in all groups (Figure 1A), indicating no treatment impact. Asthma symptoms (Figures 1B-C), including activity frequency and nose-scratching, were higher in the BA than in the Control group ($p<0.05$), but reduced in the BA+OP group than in the BA group ($p<0.05$), suggesting OP alleviated symptoms. The VDD+BA+OP group showed further symptom reduction. Airway resistance (Figure 1D) was higher in the BA than in the Control group and further increased in the BA+OP group ($p<0.05$). In the VDD+BA+OP group, resistance was slightly lower than in the BA+OP group, but not statistically significant. Cyanosis (Figure 1E) and hunched posture (Figure 1F) appeared earlier in the BA than in the Control group ($p<0.05$), even earlier in the BA+OP group, and were significantly delayed in the VDD+BA+OP group ($p<0.05$).

VDD Exacerbates Pulmonary Inflammation and Osteoporotic Lesions

The findings revealed no significant lung pathology in the Control and OP groups (Figure 1G). The BA group showed peribronchial inflammation, while the BA+OP group exhibited worsened inflammation with mucosal disruptions. The VDD+BA+OP group displayed severe lung pathology, including bronchial folds, goblet cell vacuolization, inflammation, and epithelial shedding. In bone tissues, the Control group had intact trabeculae, while the BA group showed mild thinning. The OP, BA+OP, and VDD+BA+OP groups exhibited trabecular thinning or fractures, with the most severe changes in the VDD+BA+OP group. Drug treatment outcomes showed no lung pathology in the Control group (Figure 1H). The VDD+BA+OP group had goblet cell vacuolization and epithelial shedding, the LD group showed inflammation with mucosal disruptions, and the HD group had mild inflammation. Bone trabeculae in the Control, LD, and HD groups were thicker and more intact than in the VDD+BA+OP group, which showed slight fragmentation.

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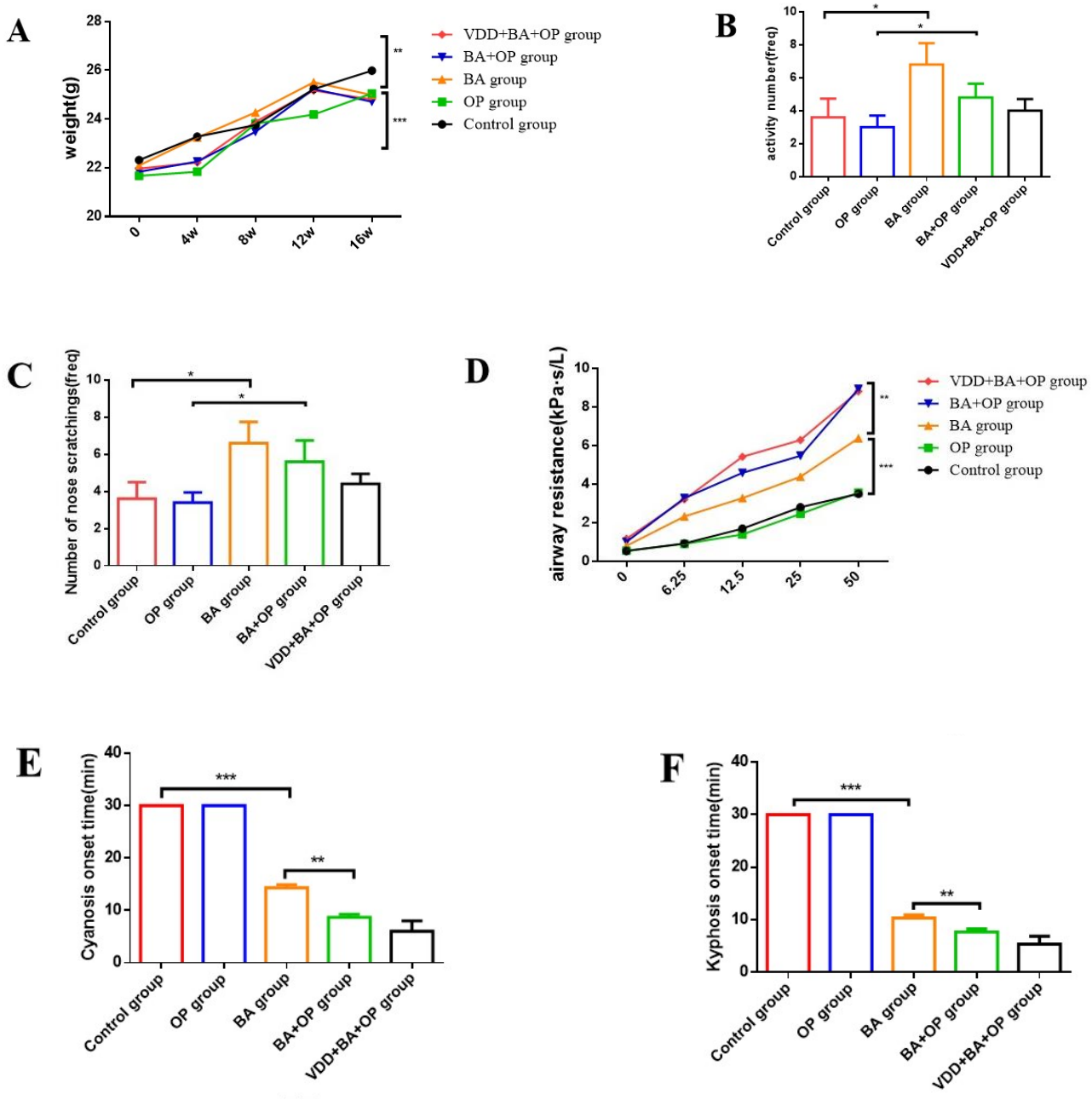
Immunohistochemical analysis (Figure 1I) revealed no significant differences in Cyp2R1, Cyp24a1, and Cyp27b1 expression among the Control, VDD+BA+OP, LD, and HD groups (p>0.05). Vitamin D binding protein (VDBP) and VDR levels were elevated in the VDD+BA+OP group, with higher VDR expression in the LD and HD groups (p<0.05). Bone tissue gene expression patterns (Figure 1J) mirrored those in lung tissues.



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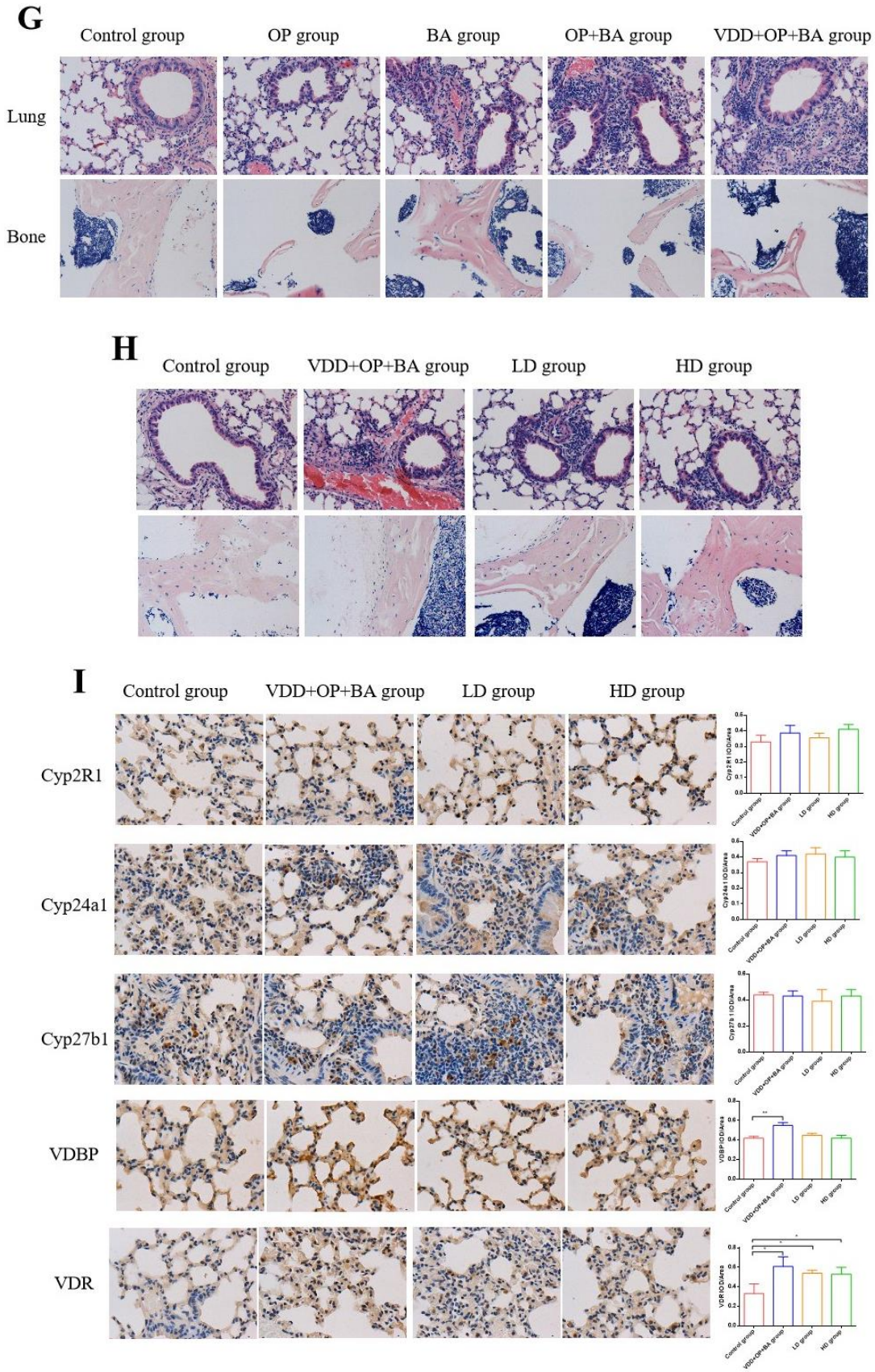

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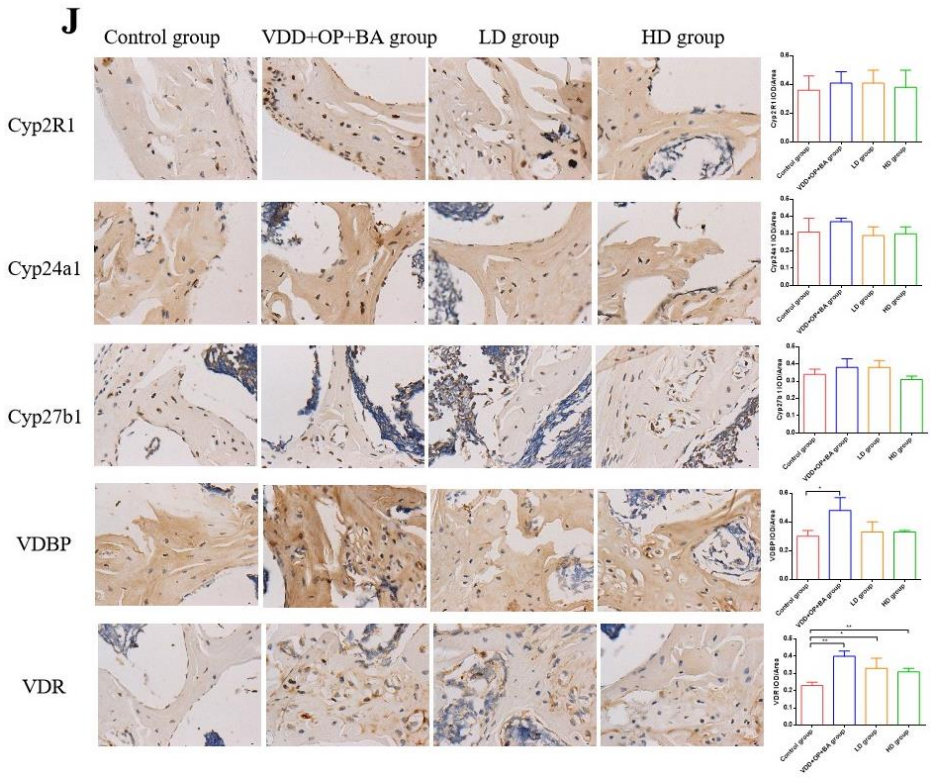


Figure 1. Effects of vitamin D deficiency (VDD) on asthma symptoms. A. Changes in body weight over time in mice from different experimental groups. B. Activity frequency in each group. C. Nose-scratching frequency in each group. D. Airway resistance in each group. E. Time to the onset of cyanosis in each group. F. Time to the onset of hunched posture in each group. G. Hematoxylin and eosin (HE) staining results of lung and bone tissues in different experimental groups. H. HE staining results of lung and bone tissues in different treatment groups. I. Immunohistochemical analysis of vitamin D metabolism-related genes (Cyp2R1, Cyp24a1, Cyp27b1, vitamin D-binding protein [VDBP], and vitamin D receptor [VDR]) in lung tissues of different experimental groups. J. Immunohistochemical analysis of vitamin D metabolism-related genes (Cyp2R1, Cyp24a1, Cyp27b1, VDBP, and VDR) in bone tissues of different experimental groups. *p<0.05, **p<0.01, ***p<0.001. VDD+BA+OP: VDD with asthma and osteoporosis group; BA+OP: asthma with osteoporosis group; BA: asthma group; OP: osteoporosis group; Control: control group; LD: low-dose group; HD: high-dose group. Scale bar: 25 μm.

VDD Impairs Bone Microstructure

Micro-CT analysis (Figures 2A-E) revealed that the Control group reflected normal bone structural integrity. In the OP and BA+OP groups, bone volume/total volume, bone mineral density, trabecular number, and trabecular thickness were significantly reduced. The VDD+OP+BA group indicates severe trabecular structure and density damage.

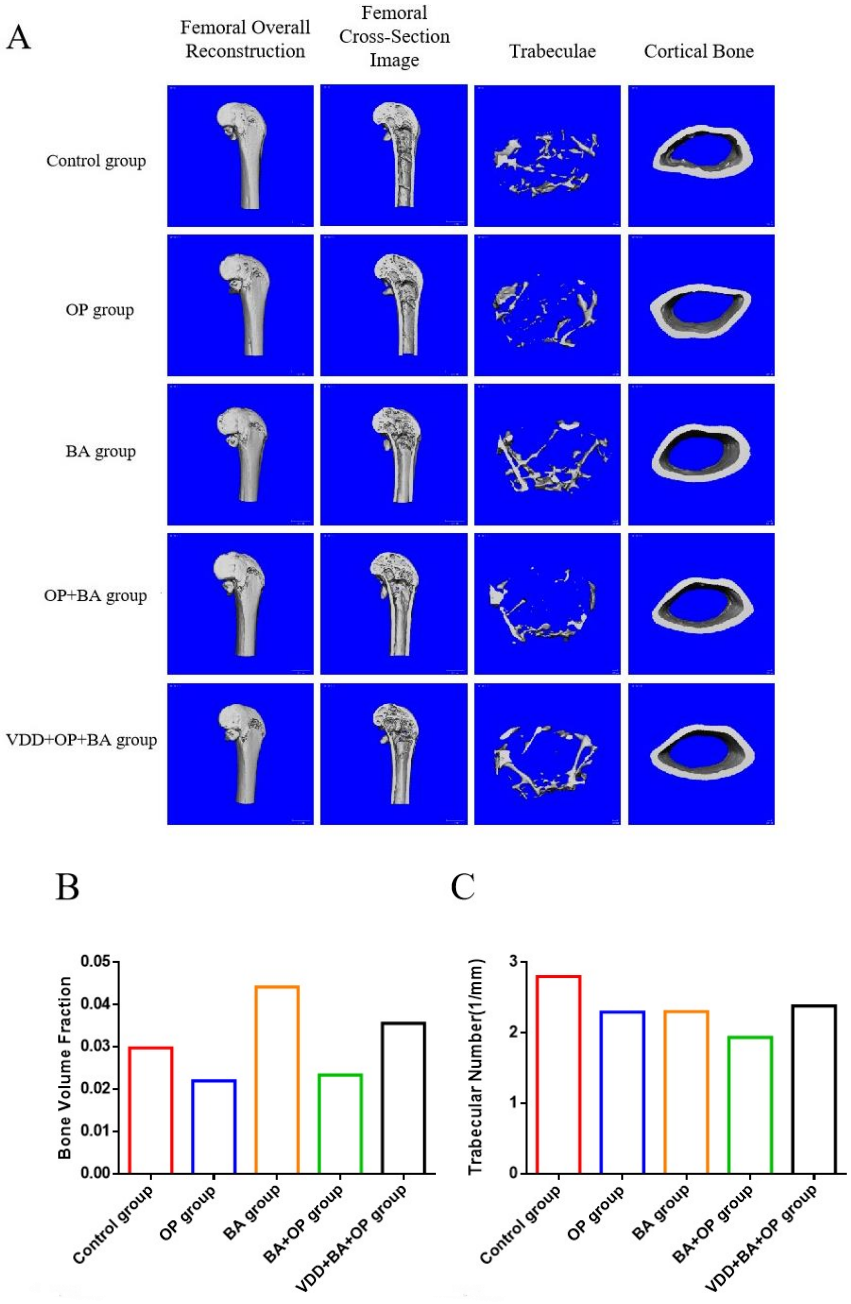
VDD Triggers Inflammatory Cytokine Release

The results (Figure 2F) showed that IL-6, IL-4, and tumor necrosis factor-alpha (TNF-α) levels significantly increased in the VDD+OP+BA group (p<0.05), while interferon-gamma (IFN-γ) levels were notably reduced (p<0.05).In contrast, IL-6, IL-17A, and IFN-γ levels significantly decreased (p<0.05) in the LD and HD groups, whereas TNF-α levels increased significantly (p<0.05), indicating that vitamin D supplementation, particularly at high doses, can suppress the release of inflammatory cytokines(Figure 2G-H). Vitamin D supplementation in the LD and HD groups led to a significant reduction in Caspase-1, N-terminal gasdermin D (NT-GSDMD), IL-1β, and IL-18 expression (Figures 2I–M). Notably, inflammatory cytokine levels in the HD group closely resembled those of the Control group (p < 0.05). Analysis of the inflammasome pathway (Figures 2N–R) showed that NLRP3, ASC, and activated Cleaved Caspase-1 expression significantly increased in the VDD+OP+BA group (p<0.05), alongside elevated IL-1β and IL-18 release (p<0.05), highlighting notable

activation of the NLRP3 inflammasome. Additionally, Caspase-1 and NT-GSDMD levels were markedly higher in the VDD+OP+BA group (p<0.05), emphasizing pyroptosis activation.

VDD Reduces Antioxidant Capacity and Enhances Oxidative Stress

The results showed that after vitamin D supplementation, glutathione (GSH) levels in the LD and HD groups significantly increased, with the HD group approaching the levels of the Control group (Figures 3A and D). For malondialdehyde (MDA) levels (Figures 3B and E), the OP, BA, BA+OP, and VDD+BA+OP were significantly elevated compared to the Control group, indicating that VDD led to increased lipid peroxidation. In contrast, MDA levels in the LD and HD groups significantly decreased. Catalase (CAT) activity (Figures 3C and F) was markedly reduced in the OP, BA, BA+OP, and VDD+BA+OP compared to the Control group, suggesting that VDD impaired antioxidant enzyme activity. Notably, CAT activity was restored in the HD group.



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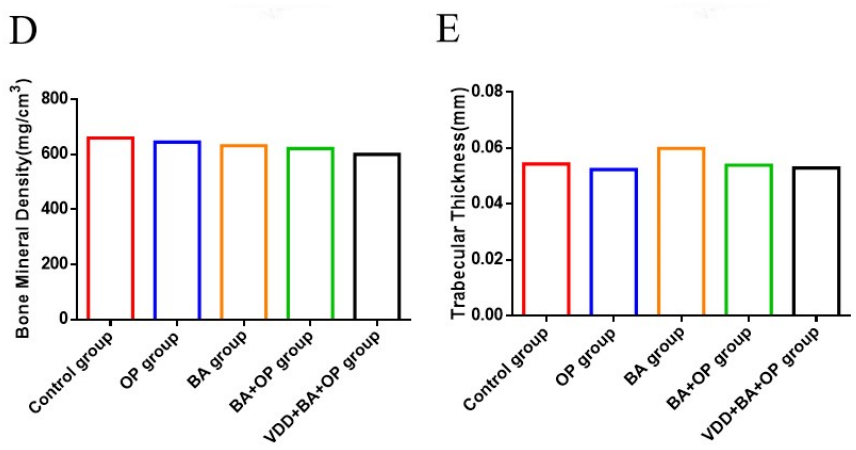
D
Bone Mineral Density(mg/cm3)
800
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200
0
Control group
OP group
BA group
BA+OP group
VDD+BA+OP group
E
Trabecular Thickness(mm)
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Control group
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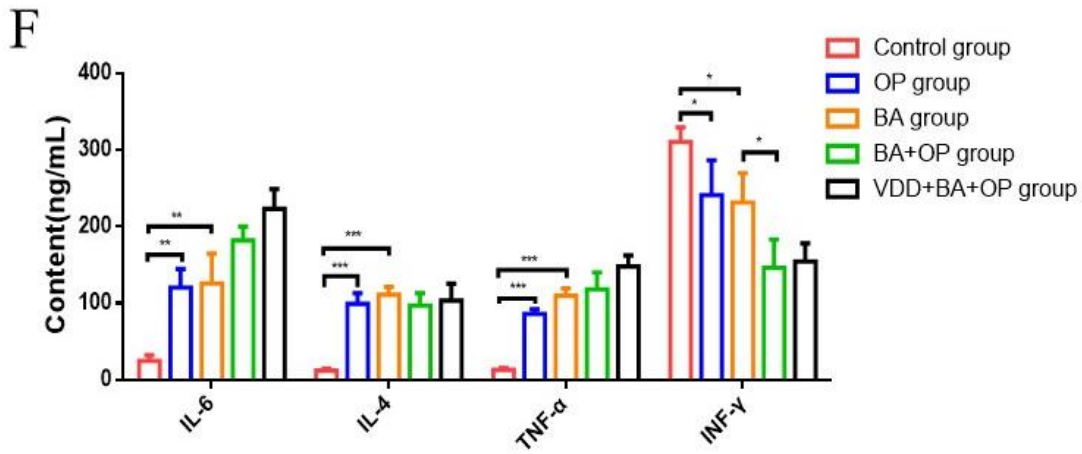
BA group
BA+OP group
VDD+BA+OP group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INF-γ
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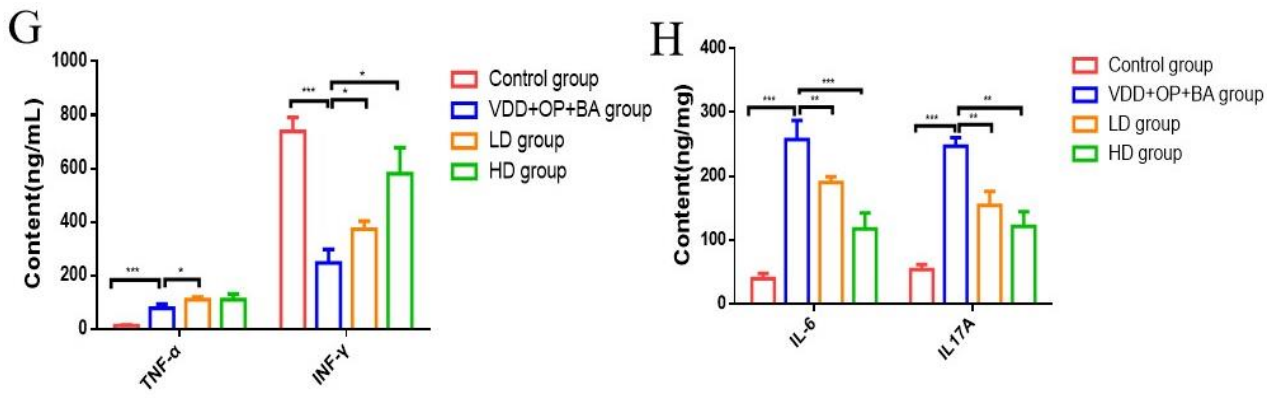
BA+OP group
VDD+BA+OP group


G
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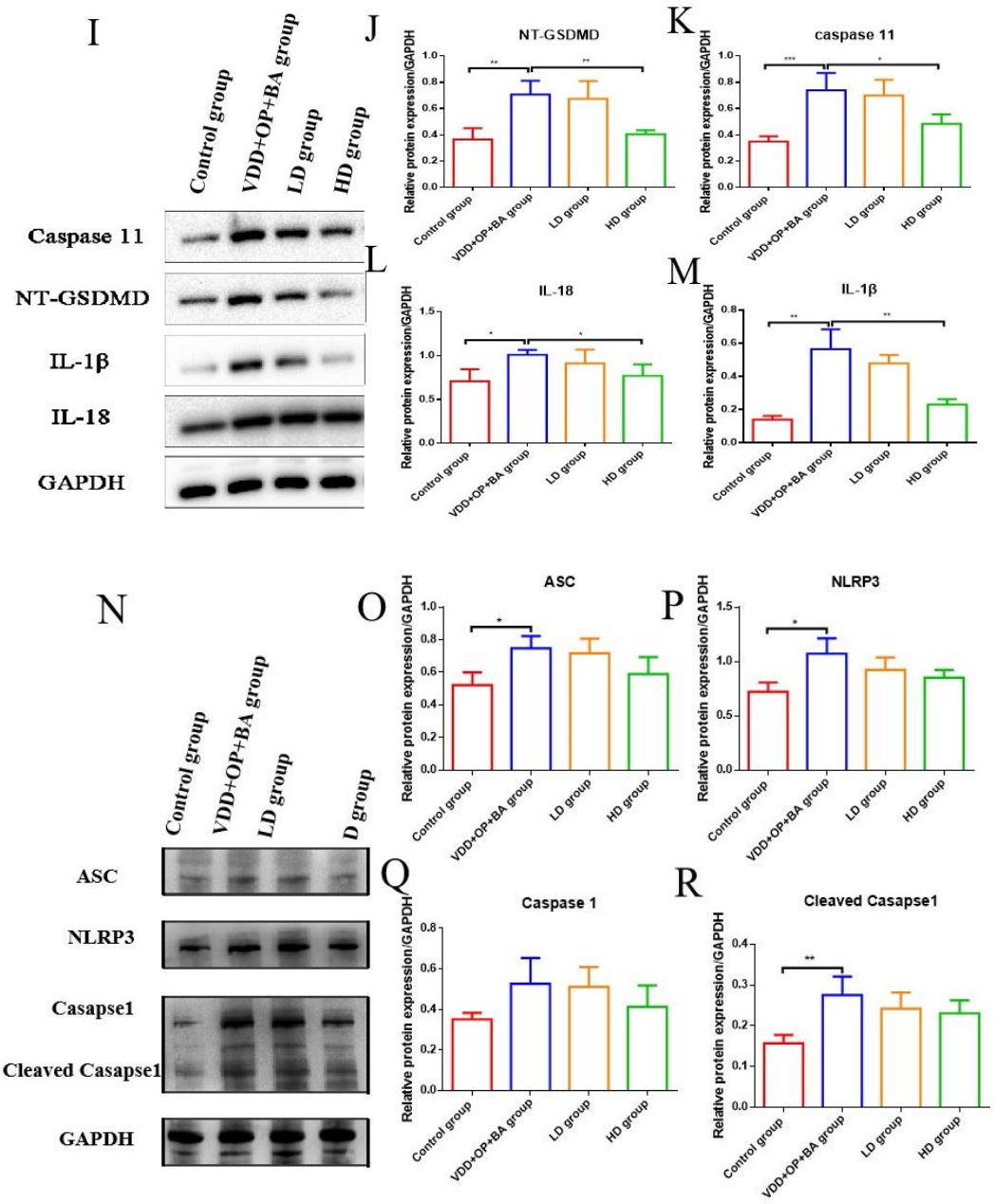

Figure 2. Analysis of bone microstructure and mechanical properties of the femur in different experimental groups of mice and the impact of vitamin D deficiency (VDD) on inflammatory cytokine levels and expression of inflammasome-related proteins. A. 3D reconstruction images of femoral micro-computed tomography (micro-CT) scans from different experimental groups. B. Bone volume fraction (BV/TV) in each experimental group. C. Trabecular number (Tb.N) in each experimental group. D. Bone mineral density (BMD) in each experimental group. E. Trabecular thickness (Tb.Th) in each experimental group. F-H. Enzyme-linked immunosorbent assay (ELISA) results showing the serum levels of interleukin-6 (IL-6), interleukin-4 (IL-4), tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and interleukin-17A (IL-17A) in different experimental groups. I. Western blot results for inflammasome-related proteins (Caspase 11, N-terminal gasdermin D [NT-GSDMD], IL-1β, and IL-18). J-M. Analysis of the relative expression levels of Caspase 11, NT-GSDMD, IL-1β, and IL-18. N. Western blot results for inflammasome-related proteins (NLR family pyrin domain-containing 3 [NLRP3], apoptosis-associated speck-like protein containing a CARD [ASC], Caspase 1, and cleaved Caspase 1). O-R. Analysis of the relative expression levels of NLRP3, ASC, Caspase 1, and cleaved Caspase 1. VDD+OP+BA: VDD with asthma and osteoporosis group; LD: low-dose vitamin D supplementation group; HD: high-dose vitamin D supplementation group; Control: control group. *p<0.05, **p<0.01, ***p<0.001.

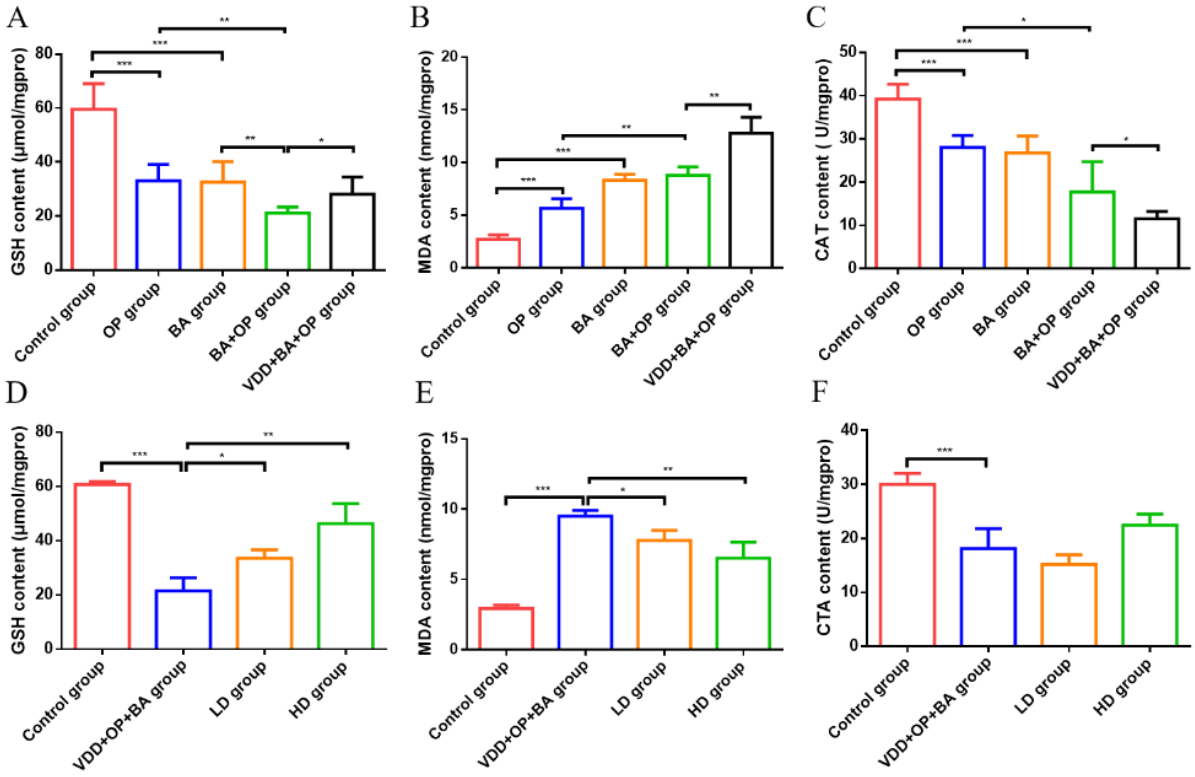

Figure 3. Effects of vitamin D deficiency (VDD) on antioxidant capacity and oxidative stress levels in mice. A and D. Serum glutathione (GSH) levels in different experimental groups detected by enzyme-linked immunosorbent assay (ELISA). B and E. Serum malondialdehyde (MDA) levels in different experimental groups detected by ELISA. C and F. Serum catalase (CAT) activity in different experimental groups detected by ELISA. VDD+OP+BA: VDD with asthma and osteoporosis group; LD: low-dose vitamin D supplementation group; HD: high-dose vitamin D supplementation group. *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Recent research has associated VDD with a higher risk of asthma.[10,11] Benerje findings revealed that VDD was more prevalent in children with asthma, with 68.1% of affected children having serum vitamin D levels≤15 ng/mL and 31.28% of infants showing insufficient levels (<20 ng/mL).[12-13] Our study suggests that osteoporosis may exacerbate asthma-related airway resistance through inflammatory mechanisms. Although airway resistance in the VDD+BA+OP was slightly lower than in the BA+OP group, this difference was not statistically significant. This indicates that the direct impact of VDD on airway resistance is limited, and its effects on inflammation or airway responsiveness might be mediated by other mechanisms. Benerje and Bosse proposed that vitamin D modulates chemokine expression in airway smooth muscle, thereby influencing airway responsiveness.[14-15]

This study demonstrated that VDD significantly worsened pulmonary inflammation, particularly in the VDD+BA+OP group, where notable bone structural changes, including thinning and fractures, were observed. However, supplementation with vitamin D led to significant improvements in both pulmonary and bone tissue conditions. Beyhan[16] also supports this view. Notably, vitamin D supplementation significantly improved forced expiratory volume in 1 second (FEV1) after 24 weeks in patients with mild to moderate persistent asthma and helped treat asthma-associated respiratory infections.[17-19] VDD can also lead to decreased bone mass, osteoporosis, and osteomalacia, increasing the risk of fractures. To address this, adequate sunlight exposure or daily intake of at least 800-1000 IU of vitamin D3 can maintain sufficient vitamin D levels. For osteoporosis treatment, combining calcium and vitamin D supplementation enhances therapeutic efficacy.[20] When serum 25-hydroxyvitamin D levels drop below 30

nmol/L, mineralization defects can be observed in iliac bone biopsies from hip fracture patients.[21-22]

The immune system plays a crucial role in asthma pathogenesis. Vitamin D modulates immune responses through various biological pathways, influencing immune system development.[23] Experimental results revealed that VDD (VDD+OP+BA group) significantly increased pro-inflammatory cytokines while reducing the anti-inflammatory cytokine INF-γ, indicating that VDD amplifies the inflammatory response. Furthermore, upstream regulatory pathways may play pivotal roles in mediating the effects of vitamin D deficiency. This aligns with findings by Zhang et al.[24] Additionally, significant activation of the NLRP3 inflammasome (elevated NLRP3, ASC, and Cleaved Caspase-1 expression) suggests that VDD exacerbates inflammation via the inflammasome pathway. This aligns with findings by Li et al.[25] Moreover, VDD significantly activated pyroptosis-related pathways, including the upregulation of Caspase-1 and NT-GSDMD[26]. In this study, elevated IL-18 and IL-1β levels were closely associated with NT-GSDMD activation, indicating that VDD promotes inflammation by enhancing pyroptosis. In contrast, the HD group markedly suppressed Caspase-1 and NT-GSDMD expression and reduced IL-18 and IL-1β levels, underscoring vitamin D's role in inhibiting pyroptosis and curbing inflammation.

VDD significantly reduced antioxidant markers such as GSH and CAT while increasing the oxidative stress product MDA, indicating impaired antioxidant capacity and elevated oxidative stress. This aligns with Lin et al.[27] In this study, heightened oxidative stress may have activated the NLRP3 inflammasome, creating a cycle of inflammation and oxidative stress. However, HD vitamin D supplementation effectively restored GSH and CAT levels, reduced MDA levels, and enhanced vitamin D's efficacy in reducing oxidative stress.

VDD exacerbates asthma symptoms and osteoporosis by increasing oxidative stress, activating NLRP3, and triggering pro-inflammatory cytokine release. The interaction between asthma, osteoporosis, and VDD creates a harmful cycle. Further research is necessary to clarify these mechanisms and provide new insights for clinical treatment.

STATEMENT OF ETHICS

This study was approved by the Animal Ethics Committee of Huzhou Central Hospital University Animal Center.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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