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Effects of Vitamin D_3 Supplementation on the Expression of *Interleukin-6* and *Vascular Endothelial Growth Factor* of Umbilical Cord-Derived Mesenchymal Stem Cells

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ABSTRACT

Mesenchymal stem cells (MSCs) were multipotent stem cells with self-renewal ability, low immunogenicity, homing, Mesenchymal stem cells (MSCs) are multipotent stem cells with self-renewal ability, low immunogenicity, homing, and immunomodulatory function; thus, they had great potential in regenerative medicine. Preconditioning of MSCs cultures is known to enhance their immunomodulatory effects in various clinical applications. This study aimed to determine the effect of vitamin D3 supplementation on the viability and expression of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) mRNA in MSCs. Methods: MSCs were isolated from Wharton's jelly umbilical cord and were cultured using the explant method. MSCs at passage 3 were cultured on 6-well plates and were divided into four experimental groups: 1, 10, and 100 nM vitamin D3 supplementation groups and the control group (without vitamin D3 supplementation). After vitamin D3 supplementation for 24 h, viability was examined using the Cell Counting Kit 8 (CCK 8) assay, and IL-6 and VEGF mRNA expression was analyzed using quantitative polymerase chain reaction (qPCR). Data were analyzed using one-way ANOVA, and p-value < 0.05 was considered significant. Results: MSCs supplemented with vitamin D3 at concentrations of 1, 10, and 100 nM for 24 h showed increased viability. Findings indicated that supplementation with vitamin D3 1 nM for 24 h significantly decreased IL-6 expression, whereas supplementation of vitamin D3 10 and 100 nM for 24 h increased IL-6 expression compared to the control group, but the difference were not significant (p=0.1883). Supplementation with vitamin D3 at 10 nM for 24 h significantly decreased VEGF expression compared to that in the control group. Vitamin D □ preconditioning modulated IL-6 and VEGF mRNA expression in UC-MSCs, suggesting potential effects on inflammatory and angiogenic pathways. Conclusion: Vitamin D □ supplementation modulated IL-6 and VEGF expression in UC-MSCs in a dose-dependent, non-linear manner.

Keywords: *Mesenchymal Stem Cells, Vitamin D3, IL-6, VEGF*

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult multipotent stem cells with self-renewal ability, low immunogenicity, homing, and immunomodulatory function, and thus have great potential in regenerative medicine, especially for tissue repair.[1,2] Mesenchymal stem cells can be isolated from various tissues, including bone marrow, umbilical cord, umbilical sord blood, adipose tissue, dental pulp, fetus and amniotic fluid.[3,4] In addition to their differentiation potential, MSCs exert therapeutic effects largely through paracrine signaling by secreting a variety of bioactive molecules collectively known as the secretome. [4,5] The secretome contains cytokines, chemokines, growth factors, signaling molecules, and anti-inflammatory mediators that contribute to immunomodulation, tissue repair, and regeneration (5,6). Among the cytokines secreted by MSCs, interleukin-6 (IL-6), plays a dual role. It regulates

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MSC proliferation, differentiation, and stemness, while also acting as a major mediator of immune responses and inflammation.[7-9] Dysregulated IL-6 expression is associated with various pathological conditions, making it a relevant target for evaluating the immunomodulatory profile of MSCs.[10-12] Another important factor secreted by MSCs is vascular endothelial growth factor (VEGF), a key pro-angiogenic molecule involved in cell migration, vasodilation, and tissue vascularization.[13-16] The combined regulation of IL-6 and VEGF contributes to the regenerative and immunomodulatory functions of MSCs.

Since the therapeutic potential of MSCs is strongly influenced by their culture conditions, strategies to optimize their functional properties are being actively investigated. Preconditioning approaches, including the use of bioactive molecules, have been shown to enhance MSC proliferation, survival, and immunomodulatory effects.[17-21] Vitamin D_3 a fat-soluble hormone with established immunomodulatory and anti-oxidative functions, exerts its cellular effects through binding to the vitamin D receptor (VDR).[22] Vitamin D □ signaling has been shown to influence MSC immunomodulatory properties, but existing studies have been limited in scope. For example, a previous study investigated vitamin D □ effects on UC-MSCs using a single concentration (50 nM) for 48 h and reported no significant change in *IL-6* expression.[23] However, the impact of different vitamin D □ concentrations and shorter exposure times on cytokine regulation in UC-MSCs has not been systematically studied. Our work was therefore designed to fill this gap by evaluating the dose-dependent effects of vitamin D □ (1, 10, and 100 nM, 24 h) on both *IL-6* and *VEGF* expression in UC-MSCs.

Umbilical cord-derived MSCs (UC-MSCs), particularly those isolated from Wharton's jelly, represent an attractive source due to their abundant availability, high proliferation rate, simple isolation procedure, and tolerance for allogeneic applications (24). However, the effect of vitamin D_3 supplementation on UC-MSC viability and cytokine expression, particularly *IL-6* and *VEGF*, remains unclear. Therefore, this study aimed to evaluate the effect of vitamin D_3 supplementation on the viability and relative mRNA expression of *IL-6* and *VEGF* in Wharton's jelly umbilical cord after 24 hours of incubation.

MATERIALS AND METHODS

This was a laboratory experimental study with a post-test-only control group design to test the effects of vitamin D_3 supplementation on the viability and expression of *IL-6* and *VEGF* in mesenchymal stem cells. Umbilical cords were obtained from mothers who gave birth with the following inclusion criteria: mothers aged 20-35 years, delivery by elective caesarean section, were not in labor, and had negative screening results for hepatitis B, hepatitis C, human immunodeficiency virus (HIV), and Covid-19 infections. The exclusion criteria were mothers who had diabetes, hypertension during pregnancy, and other complications during pregnancy, such as ante-and postpartum bleeding or fetal pregnancy. Umbilical cord specimens were collected from the Obstetrics and Gynecology Department of Hermina Hospital, Pekanbaru. Isolation and culture of MSCs and examination of the viability ang expression of growth factor genes in MSC were performed at the LONTAR Integrated Biomedical Laboratory, Faculty of Medicine, Riau University. This study was conducted from June 2024 to December 2024.

MSCs Isolation and Culture

Isolation and expansion of MSCs derived from Wharton's jelly umbilical cord. It was chopped 1-2 mm using two sterile scapels and was cultured in tissue culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM; Corning, New York, USA) with low concentration glucose, 10% fetal bovine serum (FBS; Sigma-Aldrich, Darmstadt, Germany), 100 U/mL penicillin and 100 g/ml streptomycin. The cells were cultured in an incubator with 5% CO_2 at 37^o C temperature. The first medium change was performed after 5-7 days of incubation, and non-adherent cells were removed by washing with Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Darmstadt, Germany). After the cells had developed into fibroblast-like cells and had reached ~80% confluency, they were trypsinized using 0.05% trypsin-EDTA (Sigma-Aldrich, Darmstandt, Germany) for further passaging until passaging 3-4.

Vitamin D_3 supplementation

MSCs at passage 3 were cultured in 6-well plates and were divided into four experimental groups: 1). Control group (without vitamin D_3 supplementation); 2). 1 nM vitamin D_3 supplementation (Sigma-Aldrich, Taufkirchen, Germany); 3). 10 nM vitamin D_3 supplementation; and 4). 100 nM vitamin D_3 supplementation. Each group was prepared with three replicates. After MSCs had reached ~80% confluence, vitamin D_3 supplementation was performed, and cells were were cultured in a CO_2 incubator at 37°C for 24 h. Cells were treated with vitamin D □ and were harvested at 24 h for mRNA analysis. We selected the 24 h time point because it captured stabilized transcriptional responses to receptor-mediated signaling while limited confounding effects of extended culture. This time point was commonly used in studies assessing transcriptional modulation by vitamin D and other small-molecule regulators. Previously, we had conducted preliminary studies to determine the toxicity of vitamin D □ to MSCs using various concentrations of vitamin D □ (1-100 nM) exposed for 24 h. We found that supplementation with vitamin D □ for 24 h increased MSCs viability compared to the control group.

Viability of MSCs Assessment

The viability of MSCs was assessed using the cell counting kit-8 (CCK-8) assay. Cells in passage 3 were cultured in 96-well cell culture plates at a density of $5x10^4$ per cm^2 using MSCs culture medium in a 5% CO_2 incubator at 37°C. After the cells had reached ~70-80% confluence, viability testing was performed on the experimental group using five replicates for each experiment. After incubation, the culture medium was discarded and replaced with 100 µL of serum-free medium and 10 µL of CCK-8 reagent (ABBKINE, Wuhan, China) for each well. The cells were incubated again for 3 h, and the absorbance was measured at a wavelength of 450 nm using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific). Prior to analysis we included medium-only (no cells) wells matched to each experimental condition to control for phenol red background. A well without cells was used as a blank, and 100 µL of serum-free medium and 10 µL of CCK-8 reagent were added. All sample ODs were corrected by subtracting the corresponding media/reagent blank. To validate that blank-corrected ODs reflected cell number, a standard curve of known cell numbers was prepared in the same medium/treatment conditions and showed linearity. MSCs viability was calculated using the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{OD sample-OD blank}}{\text{OD control-OD blank}} \text{ X } 100$$

Description:

OD sample = Optical Density (Absorbance) value of the tested sample

OD control = Optical Density (Absorbance) value of the control (cells without treatment)

OD blank = Optical Density (Absorbance) value of the blank (well without cells)

***IL-6* and *VEGF* Screening**

IL-6 and *VEGF* secretion was analyzed using quantitative polymerase chain reaction (qPCR). This approach was chosen to sensitively detect transcriptional changes induced by vitamin D_3 preconditioning, as our primary aim was to assess molecular regulation. After 24 h of incubation, total ribonucleic acid (RNA) was isolated from MSCs using TRNzol Universal Reagent (Tiangen Biotech, BJ, China). Synthesis of complementary deoxyribonucleic acid (cDNA) was performed by reverse transcription using 1 µg RNA and amplification of cDNA using GoTaq® 2-Step reverse transcription quantitative polymerase chain reaction (RT-qPCR) System (Promega Corporation, Madison, WI, USA). The primers used to amplify *IL-6*, *VEGF*, and *GAPDH* are listed in Table 1. RT-qPCR was performed using Heal Force X 960 RT-qPCR with a polymerase activation temperature 95°C, denaturation at 95°C, and annealing 60°C.

Table 1. qRT-PCR primer sequences

Gene	Forward	Reverse	Reference
GAPDH	GACCTGCCGTCTAGAAAAAC	TTGAAGTCAGAGGAGACCAC	25
VEGF	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCAC GAAG	26
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC	27

Data Analysis

Data with a normal distribution were presented as mean ± standard deviation (SD), and data with an abnormal distribution were presented as median (minimum-maximum). To determine the effect of vitamin D_3 supplementation on the viability and mRNA expression of *IL-6* and *VEGF* in MSCs, the data were analyzed using one-way ANOVA with post hoc Tukey's multiple comparisons test. Prior to analysis, data were assessed for normal distribution and homogeneity of variance. Statistical analysis was performed using GraphPad Prism v.9, and p value of <0.05 was considered statistically significant.

This study was approved by the Medical and Health Research Ethics Unit, Faculty of Medicine, University of Riau B/47/UN19.5.1.1.8/ UEPKK/2021_Addendum 2.

RESULTS

Effects of Vitamin D_3 Supplementation on UC-MSCs Viability

The viability of UC-MSCs after vitamin D_3 supplementation for 24 h was examined using the CCK-8 Assay, as shown in Figure 1. After 24 h of supplementation vitamin D_3 at 1 and 100 nM, the cellular viability increased compared to control group. Interestingly, cell viability showed a slight decrease at 10 nM vitamin D □ compared to the vitamin D_3 supplementation groups at concentrations 1 and 100 nM, indicating that the effect was not a simple linear increase across concentrations. Despite these changes, the statistical test did not identify significant differences between the experimental groups ($p>0.05$). These results underscored the impact of vitamin D_3 on UC-MSC viability and provided a solid foundation for further analysis of the expression of *IL-6* and *VEGF*.

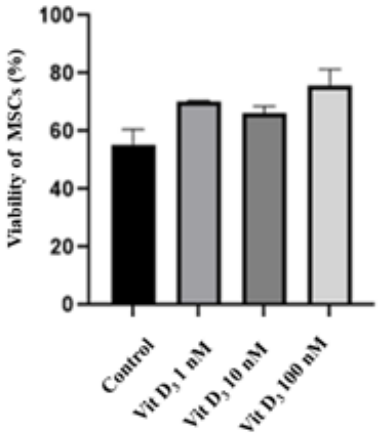

Figure 1. Effect of vitamin D_3 supplementation for 24 h on UC-MSCs viability. Mesenchymal stem cells were cultured in 6-well cell culture plates and incubated for 24 h. Cell viability was measured using the cell counting kit-8 (CCK-8) assay. Data are presented as mean ± SD (n = 5). $p<0.05$ using one-way ANOVA.

Effects of Vitamin D_3 Supplementation on *IL-6* Expression

IL-6 expression in UC-MSCs after vitamin D_3 supplementation at doses of 1, 10, and 100 nM for 24 h was examined using the RT-qPCR method for mRNA expression analysis. Each gene expression examination was normalized to *GAPDH* as a housekeeping gene. Figure 2 showed a comparison of *IL-6* expression in UC-MSCs supplemented with vitamin D_3 (1, 10, and 100 nM) compared to the control group. Supplementation with vitamin D_3 1 nM for 24 h significantly decreased *IL-6* expression compared to that in the control group ($p=0.0003$). Supplementation of vitamin D_3 10 and 100 nM for 24 h increased *IL-6* expression compared to that in the control group. However, this did not cause a significant difference compared to the control ($p=0.1883$).

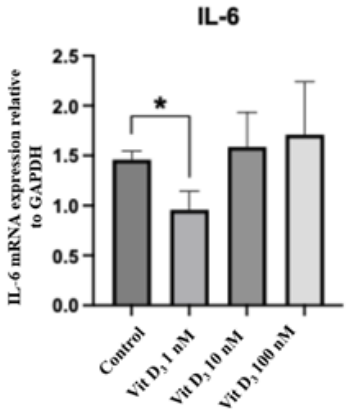

Figure 2. *IL-6* mRNA expression was analyzed using RT-qPCR and normalized to *GAPDH*. Data are presented as mean ± SD (n=3). *p<0.05 compared to control using one-way ANOVA and post hoc Tukey's multiple comparation analysis

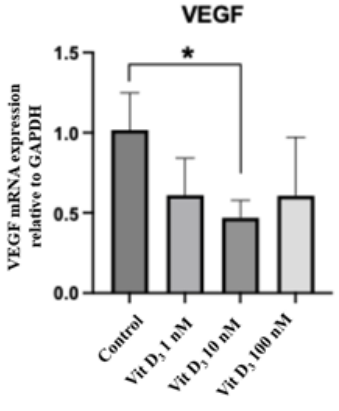

Figure 3. *VEGF* mRNA expression was analyzed using RT-qPCR, and the expression was normalized to *GAPDH***.** Data are presented as mean ± SD (n=3). *p<0.05 compared to control using one-way ANOVA and post hoc Tukey's multiple comparation analysis

Effects of Vitamin D_3 Supplementation on *VEGF* Expression

Vascular endothelial growth factor expression in UC-MSCs after vitamin D_3 supplementation at doses of 1, 10, and 100 nM for 24 h was examined using the RT-qPCR method for mRNA expression analysis (Figure 3). Each gene expression examination was normalized with *GAPDH* as a housekeeping gene. Figure 3 shows a comparison of *VEGF* expression in MSCS supplemented with vitamin D_3 (1, 10, and 100 nM) compared to the control group. Supplementation of vitamin D_3 1, 10, and 100 nM for 24 h decreased *VEGF* expression compared to the control group. After 24 h of vitamin D_3 supplementation at 10 nM concentration, a significant decreased in UC-MSCs compared to the control group ($p=0.007$). At 1 and 100 nM, *VEGF* expression levels were not significantly different from the untreated control.

DISCUSSION

Mesenchymal stem cells (MSCs) were widely studied for their applications in cell therapy, in part because of their immunomodulatory and pro-angiogenic activities, which were important for tissue repair. However, the therapeutic effects of MSCs were greatly influenced by the cell culture conditions during the in vitro expansion process. Preconditioning of MSCs cultures was found to enhance the therapeutic effects of MSCs.[28,29] Recent studies showed that vitamin D_3 could modulate transcriptional regulation of inflammatory and angiogenic pathways.[30,31] To date, the use of vitamin D_3 as a precondition in the protocol for using MSCs as a therapy remained limited. Therefore, this study aimed to determine the effect of vitamin D_3 preconditioning on the immunomodulatory properties of MSCs.

The results of the study showed that UC-MSCs supplemented with vitamin D_3 at concentrations of 1, and 100 nM for 24 h experienced increased viability compared to the control group, but not significantly. Contrary to a monotonic dose-response, cell viability showed a non-linear pattern: viability at 10 nM vitamin D □ was slightly decreased compared with control, while viability at 1 nM and 100 nM did not show a consistent upward trend. These data indicated a dose-specific, non-monotonic effect of vitamin D

□ on UC-MSC viability. The observed variability in MSC viability after vitamin D □ supplementation might have been influenced by factors such as donor-to-donor biological differences, culture conditions (including medium composition and passage number), and the sensitivity of the CCK-8 assay, in addition to the vitamin D □ concentration tested.[32] In this study, UC-MSC viability did not show a simple linear increase with higher vitamin D □ concentrations. At 10 nM, a slight decrease in viability was observed, suggesting a non-monotonic, dose-specific response. This pattern might have reflected receptor saturation, feedback regulation, or hormesis-like effects, indicating that higher vitamin D □ concentrations did not necessarily correspond to greater cell viability.

Interleukin-6 (*IL-6*) was reported as a major pro-inflammatory cytokine secreted by bone marrow–derived MSCs. Because cytokine profiles differed by MSC tissue origin, we examined whether UC-MSCs also expressed *IL-6* and whether vitamin D □ supplementation modulated this expression.[9] In our study, *IL-6* expression decreased significantly at 1 nM vitamin D □ but showed a trend toward increase at higher concentrations (10–100 nM). This biphasic response suggested that vitamin D □ might have exerted dose-dependent effects on UC-MSC immunomodulation. At lower concentrations, vitamin D □ might have enhanced the immunosuppressive profile of MSCs by downregulating *IL-6*, whereas higher concentrations might have activated compensatory or alternative signaling pathways that partially restored *IL-6* expression. Such dose-dependent, non-linear responses have been described in other vitamin D–immune interaction studies. Further investigation, including earlier and later time points as well as protein-level validation, was necessary to confirm the mechanisms underlying this observation. To date, few studies had investigated how vitamin D □ influenced cytokine expression in MSCs. *IL-6* was a central immunoregulatory cytokine secreted by MSCs, influencing their ability to modulate immune responses. Investigating the effect of vitamin D □ on *IL-6* expression in UC-MSCs provided insight into how vitamin D □ preconditioning might have altered the immunomodulatory profile of these cells. The 24-hour incubation model was chosen because it reflected a standardized preconditioning approach commonly applied in MSC studies to assess stable gene expression changes prior to potential therapeutic application.[32]

Although vitamin D □ was generally considered immunosuppressive, our study revealed that *IL-6* expression decreased significantly at 1 nM but tended to increase at higher concentrations. This apparent contradiction underscored that vitamin D □ effects on MSCs were not be strictly linear and could vary depending on dose, cellular context, and timing. Similar paradoxical responses had been reported in receptor-mediated signaling systems, where high ligand concentrations could induce receptor desensitization or alternative pathway activation. These findings suggested that vitamin D □ might have exerted a dual modulatory effect on UC-MSCs, and highlighted the need for additional studies including multiple time points and protein-level validation. Our findings indicated that vitamin D □ supplementation modulated the transcriptional expression of *IL-6* in UC-MSCs. While *IL-6* was a pro-inflammatory cytokine, these changes were observed only at the mRNA level and should have been interpreted cautiously. We did not evaluate VDR expression, promoter interactions, or signaling pathways; therefore, mechanistic conclusions could not be drawn from this study. Instead, our results provided preliminary evidence that vitamin D □ might have altered the molecular profile of UC-MSCs in a dose-dependent manner, highlighting the need for further protein-level and functional validation. Our previous work reported that 50 nM vitamin D □ for 48 h did not significantly affect *IL-6* expression in MSCs. Although the study used a different concentration and longer incubation period than ours, both sets of findings suggested that vitamin D □ did not exert a uniform, linear effect on *IL-6* expression. Instead, the outcome appeared to depend on multiple factors, including dose and exposure time, which might have explained the divergent responses observed between studies.[23,32]

Vascular Endothelial Growth Factor (VEGF) was a growth factor secreted by human umbilical cord MSCs. Vascular Endothelial Growth Factor could stimulate angiogenesis and enhance the therapeutic effects of MSCs.[33] The results of this study showed that the administration of vitamin D_3 to UC-MSCs led to a decrease in *VEGF* expression. The results of another study that

used hypoxic preconditioning in MSCs showed an increase in *VEGF* expression.[29] In this study, 1, 10, and 100 nM vitamin D_3 preconditioning decreased *VEGF* expression. Supplementation of vitamin D_3 10 nM for 24 h significantly decreased *VEGF* expression compared to the control group. Similarly, supplementation with 50 nM vitamin D_3 for 48 h was shown to significantly decrease *VEGF* mRNA levels compared to the control group.[23] This suggested that vitamin D_3 might have modulate the inflammatory and pro-angiogenic properties of MSCs, potentially inhibiting the *VEGF* production.[32] The ability of vitamin D_3 to modulate the angiogenic potential of MSCs might have had therapeutic implications for tissue regeneration and wound healing. Although *VEGF* expression was decreased at certain concentrations, vitamin D □ might still have supported angiogenesis indirectly, for example through its immunomodulatory effects that created a more permissive environment for vascular repair. Therefore, vitamin D □ preconditioning could potentially have enhanced the effectiveness of MSC-based therapies for vascular injury, but further functional studies (e.g., migration or wound-healing assays) were required to confirm this hypothesis.[23,32]

Our findings indicated that vitamin D □ preconditioning modulated *IL-6* and *VEGF* expression in UC-MSCs in a non-linear, dose-dependent manner. *IL-6* was significantly reduced at 1 nM but tended to increase at higher concentrations, whereas *VEGF* expression decreased only at 10 nM. Such biphasic responses were consistent with receptor-mediated signaling, where feedback regulation, pathway crosstalk, or cellular heterogeneity could have influenced transcriptional outcomes. Interestingly, vitamin D □ supplementation enhanced UC-MSC proliferation while *VEGF* expression was reduced at the 24-hour interval, suggesting dynamic regulation in which *VEGF* might have been transiently upregulated during early phases and later suppressed as proliferation predominated. Because this study assessed only mRNA levels at a single time point, the biological relevance of these changes remained to be clarified. Future studies incorporating multiple time points, intermediate doses, and protein-level as well as functional analyses would be crucial. Nonetheless, these preliminary data suggested that vitamin D □ could have altered the molecular profile of UC-MSCs, offering insight into strategies for optimizing their therapeutic application.

CONCLUSION

Vitamin D □ supplementation modulated *IL-6* and *VEGF* expression in UC-MSCs in a dose-specific, non-linear manner, suggesting its potential as a preconditioning agent. These preliminary findings highlighted the need for further studies with broader dose ranges, multiple time points, and protein-level validation to confirm its therapeutic relevance. The observed decrease in *IL-6* expression at lower concentrations suggested potential modulation of inflammatory pathways, while the reduction of *VEGF* at specific doses highlighted a complex regulatory effect on angiogenesis. These findings indicate that vitamin D □ preconditioning might have altered the immunomodulatory and regenerative properties of MSCs, providing new insights into optimizing their therapeutic potential.

A key limitation of this study was that the analyses were conducted at a single 24-hour time point and were limited to the assessment of mRNA expression levels. Transcriptional alterations might not necessarily have translated to corresponding changes at the protein level or functional outcomes. Moreover, dynamic or transient responses that might have occurred at earlier or later intervals could not be captured within this design. In addition, the study did not include functional assays such as cytokine secretion, cell migration, proliferation, or angiogenesis models, which would have been essential to confirm the biological significance of the observed molecular findings. Therefore, while the data provided valuable preliminary insights into the effect of vitamin D_3 on MSCs, further investigations incorporating protein-level validation and functional assessments were required to establish the mechanistic and therapeutic relevance of these results.

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