ORIGINAL ARTICLE

# Calcitriol attenuates inflammatory response in the lung of diabetes mellitus rat model

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## ABSTRACT

**Introduction: Inflammation caused by diabetes can damage multiple organs, including the lungs. Vitamin D (VD) has been shown to potentially reduce inflammation and boost the immune system. VD might play a role in diabetes' inflammatory response. This study aims to elucidate the evidence regarding the lung as the target organ for DM and the possible role of VD in preventing pulmonary damage progression in the diabetes rat model.**

**Material and Methods: Thirty Sprague Dawley rats (3-month-old, 200 to 300 gm) were randomly divided into six groups, namely control (C), 4 weeks diabetes mellitus (DM1), 8 weeks DM (DM2) and three DM1 groups (VD1, VD2, and VD3) who received Vitamin D doses of 0.125, 0.25 and 0.50 µg/kg BW, respectively. After 4 weeks, daily VD was administered intraperitoneally for 30 days. Lung tissues were taken for IL-6, MCP-1, NFKB and CD68 mRNA expression analysis and paraffin embedding. Immunohistochemical staining against CD68 and MCP-1 was conducted. Data were analysed using one-way ANOVA. $p < 0.05$ was considered statistically significant.**

**Results: DM2 group represented significantly higher IL6, MCP1, NFKB and CD68 mRNA expression than Control group ($p < 0.05$). Meanwhile, VD2 and VD3 groups revealed significantly lower mRNA expression of IL-6, MCP1, NFKB and CD68 than DM2 ($p < 0.05$). Immunostaining revealed the spreading of MCP1 protein expression in lung tissue along with macrophage infiltration in the DM2 group, which was reduced in the VD2 and the VD3 groups.**

**Conclusion: VD shows a protective effect on diabetes-induced lung damage by regulating inflammation factors.**

**KEYWORDS:**
*Vitamin D, calcitriol, lung, diabetes, inflammation*

## INTRODUCTION

Diabetes mellitus (DM) is a chronic systemic disorder associated with many complications in various organs through neurological, microvascular and macrovascular damage.[1] The complex alveolar-capillary network of the lungs may be affected through diabetic microvascular damage. However, there is lack of study regarding diabetes effects in lungs tissue.[2-4] DM is associated with architectural and functional damage of lungs tissue.[4-6]

DM leads to various pathological responses one of which begins with an inflammatory process involving inflammatory cells, cytokines, chemokines and signalling pathways such as receptors of advanced glycation end-products (RAGE), janus kinase/signal transducer and activator of transcription (JAK/STAT), NFKB. The condition leads to end organ injury and damage as final consequences on DM. Excessive inflammation response in the lungs leads in deleterious effects as impaired lung function.[2,3] Patients with diabetes has been reported in increased risk of having lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPD), pulmonary hypertension, cancer as well as infections.[2] Effects of diabetes conditions in end organ injury may give insight into prevention of diabetic progression and reducing the implication of diabetes mellitus, especially in lung injury.

Many substances have beneficial effects in inflammation, such as Vitamin D (VD). VD has been shown to potentially reduce inflammation and boost the immune system. Several experimental studies have shown that VD has an antioxidant effect by inhibiting the production of free radicals and oxidative modification of other biomolecules.[7] VD plays a role in inhibiting the chemotactic effect of monocytes/macrophages in inflamed tissues.[8] Previous study demonstrated calcitriol 0.05 µg/mL/100 gm BW/day attenuated inflammation and fibrosis in chronic kidney diseases model in rats.[9] Focusing on effects of diabetes condition on end organ injury, especially lung, VD might play a role in diabetes' inflammatory response, especially in lung tissues. This study aims to elucidate the evidence regarding the lung as the target organ for DM and the possible role of VD in preventing pulmonary damage progression in the diabetes rat model.

## MATERIALS AND METHODS

### Study Design and Diabetes Mellitus Induction

This quasi-experimental study had been approved by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing,

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**Table I: Average blood glucose levels pre and post vitamin D (VD) treatment**

| Groups | Pre-VD Mean ± SD | Post-VD Mean ± SD | p-value |
|---|---|---|---|
| Control | 111.4 ± 8.02 | 111 ± 9.43 | 0.807 |
| DM1 | 379.2 ± 88.73 | 556.2 ± 126.88 | 0.043* |
| DM2 | 420.2 ± 94.61 | 566.2 ± 96.85 | 0.071 |
| VD1 | 478.25 ± 100.83 | 308.75 ± 81.06 | 0.005* |
| VD2 | 362.8 ± 58.67 | 301.3 ± 81.87 | 0.245 |
| VD3 | 414.6 ± 55.16 | 292.58 ± 87.34 | 0.144 |

DM1 = 1 month diabetic group; DM2 = 2 months diabetic group; VD1 = DM2 group treated with VD 0.125 µg/kgBW; VD2 = DM2 group treated with VD 0.25 µg/kgBW; VD3 = DM2 group treated with VD 0.5 µg/kgBW. *p < 0.05 post VD vs. Pre VD.

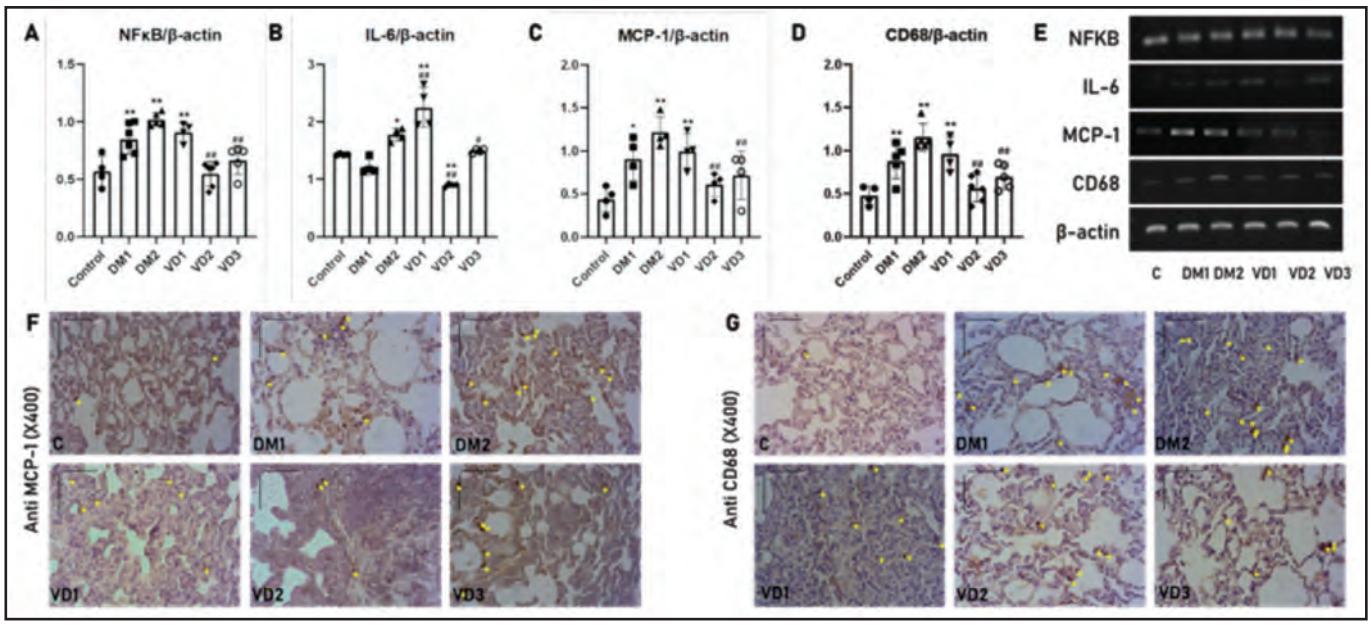


**Fig. 1:** (A D). NFKB, IL-6, MCP-1 and CD68 mRNA expression. *p < 0.05 vs control, **p < 0.01 vs control, #p < 0.05 vs DM2, ##p < 0.01 vs DM2. (E). Representative picture of anti-MCP-1 IHC staining with 400x magnification. The yellow triangle indicates the positive staining. (F). Representative picture of Anti-CD68 IHC staining with 400x magnification. The yellow triangle indicates the positive staining.

Universitas Gadjah Mada with the ethical clearance number KE/FK/1057/EC/2022. A total of 36 male Sprague Dawley rats (3-months-old, 200 to 300 gm) were randomly divided into six groups: control (C), DM 1 month (DM1), DM 2 months (DM2) and three variations of DM2 groups injected with several dosages of VD as the treatment groups. All rats were caged on a 12:12 hour's light-dark cycle under controlled conditions and given free access to standard diet and tap water.

The DM was induced by single intraperitoneal (i.p.) injection of streptozotocin (STZ) (13104, Cayman chemical) 60 mg/kg body weight (BW) dissolved in 0.1 M citrate buffer pH 4.5. Blood glucose level was quantified on day 6 post induction from the tail vein (pre-VD). DM was defined if the blood glucose level (BGL) was higher than 250 mg/dL. Rats that received the same amount of single i.p. injection of the solvent were used as control group (C) and euthanised after 2 months.

**Calcitriol Administration**

Diabetic conditions was performed in the rats for 1 month, then the rats with diabetic condition (blood glucose > 250 mg/dL) were randomised, and treated with Calcitriol in a month. After 1 month of DM induction, i.p. injection of VD in the form of calcitriol (71820, Cayman Chemical) dissolved in 1 ml of 0.2% ethanol were given for the treatment groups. The i.p. injection was administered once daily between 10 to 12 a.m. for 30 consecutive days. Three variations of the treatment groups based on the previous study (9) were VD1 (DM2 group treated with VD 0.125 µg/100 gm BW), VD2 (DM2 group treated with VD 0.25 µg/100 gm BW), and VD3 (DM2 group treated with VD 0.05 µg/mL/100 gm BW) per day. Higher dose was similar with our reference dose based on our previous study.9 The DM2 group also received the same amount of solvent during the same period.

**Termination**

Prior to euthanasia, the BGL of all rats was quantified (Post-VD). The rats in the DM1 group (after 6 weeks) and all other groups (after 8 weeks) were sacrificed by i.p. ketamine

injection (KTM100, PT. Bernofarm Pharmaceutical, Indonesia) at a dose of 100 mg/kgBW. After deep anaesthesia, the abdomen and thorax were then opened, and the left ventricle was perfused with phosphate buffer saline to clear the red blood cells from the lungs. The proximal bronchus of right lung was tied tightly and then the right lung lobes were carefully removed distal from the knot. A 22-G angiocath was then inserted and tied into lung trachea, through which neutral buffered formalin (NBF) was instilled with the pressure of 25 cmH2O. Lungs were then removed and immediately stored in 300 µl of FavorPrep™ RNA stabilization solution (part number: FARSS 001, Favorgen®, Taiwan) at -80oC for RNA extraction and neutral buffered formalin for paraffin embedding.

**RNA Extraction, cDNA Synthesis and Reverse Transcriptase PCR**

RNA from the lung tissues was isolated using FavorPrep™ Tri-RNA Reagent (FATRR 001, Favorgen® Biotech Corporation) according to the manufacturer's protocol. After RNA concentrations were quantified using a nanodrop, the cDNA was synthesised using ExcelRT™ Reverse Transcription Kit II, 100 Rxn (part number: RP1400, Smobio Technology, Inc., Taiwan). cDNA samples were then stored at -20°C. The cDNA was used for mRNA expression quantification using RT-PCR. These following primers were used for the RT-PCR: NFKB (forward: GCCTGACACCAGCATTTGA; reverse: CAAACCAAACAGCCTCACG), IL-6 (forward: TCCTACCCCAACTTCCAATGCTC; reverse: TTGGATGGTCTTGGTCCTTAGCC), MCP-1 (forward: GCTGTAGTATTTGTCACCAAGCTC; reverse: ACAGAAGTGCTTGAGGTGGTT), CD68 (forward: TGTGTCCTTCCCACAAGCAG; reverse: AAGAGAAGCATGGCCCGAAG), and β-actin (forward: GCAGATGTGGATCAGCAAGC; reverse: GGTGTAAAACGCAGCTCAGTAA), followed by electrophoresis procedure. Gene expression was quantified with densitometry analysis using ImageJ® software and normalised by ß-actin.

**Immunohistochemical (IHC Staining)**

After deparaffinisation, rehydration and antigen retrieval, the sample slides were douse with 3% H2O2 in PBS for endogenous peroxidase inhibition and incubated in blocking solution using Mouse/Rabbit Probe HRP Labeling Kit with DAB Brown IHC kit (Catalog number: TAHC03D-100; BIOTnA®). The slides were then incubated at 4°C overnight with primary antibody overnight, including anti CD-68 (ab955, 1:100, Abcam) and anti MCP-1 (ab25124, 1:300, Abcam). Then, the slides were incubated with species specific secondary antibody provided by the IHC kit at room temperature for 1 hour, followed by incubation with a DAB working solution. Haematoxylin was then used for re-staining. Examination across the entire visual field of the lung tissue was done under a light microscope (at least 400 × magnifications). A descriptive analysis was performed by comparing representative images from all study groups.

**Data Analysis**

The data obtained were analysed using IBM SPSS Statistics for Windows, version 26. Each parameter was described as the mean value ± standard deviation (SD). The data distribution of numeric data was tested with the Shapiro-Wilk test. One-way analysis of variance (ANOVA) test and post hoc least significant difference (LSD) test was used. Paired T-test was used to compare blood glucose level before and after VD administration. The value of $p<0.05$ was considered statistically significant.

## RESULTS

**Blood Glucose Level**

After injection of STZ, the BGL of diabetic rats in our study ranged from 252 mg/dl to 585 mg/dl which indicated a successful model. BGL were taken before VD treatment (pPre-VD) and after VD treatment (post-VD) (Table I). BGL before VD treatment showed significant difference between all the diabetic groups with control group ($p < 0.05$). All VD treated groups had significant lower glucose level compared with both DM groups ($p < 0.05$), but not as low as the control group.

**Diabetic Rats Showed More Severe Inflammatory Response than Non-Diabetic Rats**

To explore the different inflammatory response between groups, we performed RT-PCR for inflammatory response-related genes and IHC staining for protein (Figure 1). The overall gene expressions, as shown in NFKB, IL-6, MCP-1, and CD68 mRNA expressions, exhibited significantly higher inflammation in diabetic rats, especially in DM2 group ($p < 0.05$), than in the control group (Figure 1A E). This data is supported by higher protein expression of MCP-1 (Figure 1F) and CD68 (Figure 1G). The anti-MCP-1 staining of the lung alveolar epithelium and interstitial tissue and anti-CD68 staining revealed higher macrophage cell infiltration and ultimately inflammatory reaction in rats with hyperglycaemia.

**Calcitriol Attenuated the Inflammatory Responses in Diabetic Rats**

To assess the optimum dose of calcitriol to prevent the progression of inflammatory in diabetic rats, we gave daily i.p. injection of calcitriol in three different dosages. The VD treated groups showed lower mRNA expressions of NFKB, IL-6, MCP-1 and CD68 compared to DM2 group (Figure 1A E), especially in group VD2 ($p < 0.01$) and VD3 ($p < 0.05$). Changes of inflammatory response were also detected in IHC staining of anti-MCP-1 and anti-CD68 in the lung tissues (Figure 1F G). The results indicated a marked decrease of inflammation in the VD treated groups, especially in dose 0.25 µg/kgBW and 0.5 µg/kgBW.

## DISCUSSION

This study demonstrated that calcitriol treatment in diabetic conditions may ameliorate lung tissue injury and reducing inflammation responses. After administration of STZ, all of the diabetes groups had BGL results of over 250 mg/dl. This indicates the success of the diabetes induction.[10] The DM2 groups that were treated with VD showed a decrease in the BGL after a month of daily i.p. injection, especially VD 1 group. VD is hypothesised to exhibit glycaemic control property.[11,12] Circulating active form of VD, calcitriol, directly affects pancreatic β-cell function by binding to the vitamin D

receptor (VDR) on its membrane. It regulates the flow of calcium through the membrane in the pancreatic β cells, increasing insulin secretion and preventing apoptosis of pancreatic β cells as not to exacerbate DM. It also regulates the flow of calcium on insulin target cells in peripheral tissues and preventing occurrence of insulin resistance, thereby reducing BGL.[13] This property might be beneficial especially in diabetes patients with deficient VD status.12 Therefore, VD deficiency is thought to be the risk factor for development of diabetes.[14,15]

For a long time, the clinical significance of the relationship between diabetes and lungs was not well understood. However, Schuyler et al in 1976 were among the first to report the debilitating effect of diabetes in lung function.[2,5] Hyperglycaemia has been shown to be associated with airway inflammation.[2] In hyperglycaemia state, advanced glycation end-products (AGE) are actively produced and accumulate in the circulation and various tissues such as endothelium, smooth muscle cells, cardiomyocytes, neural tissue, and mononuclear cells.[16] Receptor for advanced glycation end-products (RAGE) is highly expressed in the pulmonary endothelium, bronchial and vascular smooth muscle, as well as in alveolar macrophages.[2,17] AGE increases the expression of RAGEs. Upon recognition of AGE by RAGE, oxidative stress production was accelerated in cells and then causes an inflammatory response through activation of NF-κB. NF-κB induced secretion of various cytokines and growth factors such as IL-6, IL-1α, TNF-α.[16,18,19]

Our study revealed upregulation of inflammatory mediator and macrophage marker CD68 in lung in diabetic condition, which showed complication of diabetes in lung inflammation. This downstream pathway of inflammatory response also causes increased in chemokines such as monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes to inflammation sites.[2,30] This chemokine has been shown to predict a poorer prognosis in diabetes patients with asthma.[21] The macrophage infiltration could be detected by increased in cluster of differentiation 68 (CD68) in lung tissues.[21] Thus, inflammatory signalling which involving NF-κB and its downstream such as IL-6 and MCP-1 play role in inflammatory responses in the lung after diabetic condition. This increase in inflammatory response could be observed in our non-treated diabetic groups which heighten along with duration of hyperglycaemia. In previous diabetic mice model, there are association between diabetes duration with kidney injury and MCP-1 expression in kidney and urine.[23]

Inflammatory response could exacerbate the tendency of thrombosis, leading to the development of arteriosclerosis in micro and macrovascular pathway of chronic hyperglycaemia complication.[15,24] In endothelial cells, the AGE-RAGE system triggers nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and accelerates oxidative stress which causes endothelial dysfunction.[25] Endothelial dysfunction also causes an increase in MCP-1 and macrophage infiltration which further exacerbates the inflammation response.[22]

Our study highlights the effect of Vitamin D in downregulating inflammatory mediators signalling, especially NF-κB, IL-6 and MCP-1. We observed significantly lower pro-inflammatory factors in VD treated groups, especially VD 2 and VD 3 to DM2 groups. Vitamin D is a known anti-oxidative and immunomodulator. VD could lower the inflammation cytokine transcription through NFκB and mitogen-activated protein kinase phosphatase-1 (MKP-1) pathway.[26] VD also lowered monocyte/macrophage chemotactic through increasing expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) which hinder the phosphorylation of NFkB p65, p38 MAPK and Erk1/2 in human adipocyte.[8] This immunomodulator features of VD in various organs of diabetes rat model such as liver,[27] kidney,[28] gingival epithelium,[29] and also pancreas.[30] The mRNA expression of MCP-1 and CD68 is supported with the histological findings. We observed lower MCP-1 and CD68 positive staining in VD treated groups compared to DM2 group. These findings could indicate the protective effect of VD through attenuating the inflammatory response in the lung tissues from DM. Quantification of protein level for assessing inflammatory mediator using western blotting or ELISA may give better understanding for this study, and it's may become limitation of the study. Quantification of macrophage infiltration may be important also for the next study. This result also may give perspective in application of VD in diabetic condition, both in pre-clinical or clinical setting which may give better outcome. However some additional data for functional analysis and more comprehensive quantification from both transcriptomic and proteomic analysis are needed for better understanding.

## CONCLUSION

This study demonstrated that VD may ameliorate inflammation in the lung in diabetic condition. Adding lung function assessment and elucidating the possible stress oxidative pathway and fibrosis as the end-result of chronic inflammatory reactions may provide a better understanding about the potency of VD to prevent diabetes complications in lungs tissue.

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