RESEARCH ARTICLE

Vitamin D and Magnesium Combination Lowers High-Sensitivity C-Reactive Protein, Interleukin-12, Renin, and Carotid Vessel in Diabetic Rats

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Received date: Feb 20, 2026; Revised date: Apr 11, 2026; Accepted date: Apr 15, 2026

Abstract

BACKGROUND: Diabetes mellitus induces oxidative stress, inflammation, and endothelial dysfunction, accelerating atherosclerosis development marked by elevated high-sensitivity C-reactive protein (hs-CRP) and interleukin-12 (IL-12). Diabetes often causes significant vascular changes, which can be represented by carotid tunica intima thickening and elevated renin level. Metformin, which remains as the standard therapy, has limited vascular effects. Vitamin D and magnesium exhibit vasoprotective and anti-inflammatory properties; however, their combined effects on diabetic vascular injury remain unclear. This study evaluated the effects of vitamin D and magnesium combination on serum hs-CRP, IL-12, renin, and carotid tunica intima thickness in diabetic rats.

METHODS: Wistar rats were diabetic-induced by 45 mg/kg streptozotocin and 110 mg/kg nicotinamide. Following the induction, the rats were subsequently assigned to receive no treatment, metformin, or a combination of vitamin D and magnesium. Healthy rats were also included as control. Blood sample was collected after 21 days of treatment. Serum hs-CRP, IL-12, and renin were measured using enzyme-linked immunosorbent assay (ELISA), while the carotid tunica intima thickness was assessed by Doppler ultrasound.

RESULTS: Diabetic rats showed significant increases hs-CRP, IL-12, renin level, and intima thickness (p<0.001) compared to control. Both metformin and combination of Vitamin D and magnesium reduced these parameters, with combination of Vitamin D and magnesium showing the greatest improvement. Carotid intima thickness in the combination of Vitamin D and magnesium group was comparable to normal controls, indicating marked vascular protection.

CONCLUSION: Vitamin D and magnesium combination provided superior glycemic, anti-inflammatory, and vascular protective effects compared to metformin alone. This adjunctive strategy might have potential in preventing vascular complications in diabetes.

KEYWORDS: diabetes mellitus, oxidative stress, endothelial dysfunction, hs-CRP, IL-12, atherosclerosis

Indones Biomed J. 2026; 18(2): 193-200

Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycemia.(1) It remains a major cause of morbidity and mortality worldwide, primarily due to its vascular complications. One of its long-term consequences is atherosclerosis, the underlying mechanism of coronary heart disease (CHD), which ranks as the second leading cause of death among Indonesians aged 30–70 years.(2) In Indonesia alone, an estimated 8.2 million people live with diabetes mellitus.(3)

Persistent hyperglycemia induces endothelial dysfunction, oxidative stress, and inflammation, all of which are central to the initiation and progression of atherosclerosis.(4) Reactive oxygen species (ROS) accelerate low-density lipoprotein (LDL) oxidation, which triggers activation of endothelial and smooth muscle cells as well as macrophages, leading to the release of pro-inflammatory cytokines such as interleukin (IL)-12 and IL-6.(5) These cytokines, particularly IL-6, IL-1β, and IL-17, stimulate C-reactive protein (CRP) synthesis, resulting in elevated circulating CRP.(6) In oxidative acidic environments, CRP binds to atherogenic LDL, enhancing vascular inflammation and plaque formation.(7) Furthermore, hyperglycemia reduces nitric oxide (NO) bioavailability, impairing vasodilation and promoting endothelial injury.(4) Collectively, these processes contribute to thickening of the carotid intima-media layer, an early marker of atherosclerosis in diabetes mellitus.(8) ROS itself interacts synergistically with metabolic changes to induce pathological activation of renin-angiotensin-aldosterone system (RAAS).(9) Therefore, renin level was found high in hyperglycemia-state, which in turn worsening insulin resistance and disrupting glucose haemostatis.(10)

Although metformin remains the first-line pharmacologic therapy for type 2 diabetes mellitus, its direct effects on vascular inflammation are limited. Therefore, exploring adjuvant interventions that combine metabolic control with vascular protection is clinically relevant. Vitamin D has been recognized for its vasoprotective and anti-inflammatory effects through stimulation of NO production and suppression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity.(11) Magnesium also contributes to vascular health by acting as a natural calcium antagonist, mitigating oxidative stress, and modulating inflammatory pathways.(1) However, the effects of vitamin D and magnesium combination on inflammatory markers, renin, and carotid intima thickness in diabetic rat models have not been well elucidated. Therefore, this study was conducted to evaluate the effects of combined vitamin D and magnesium supplementation on serum hs-CRP, IL-12, renin, and carotid tunica intima thickness in streptozotocin–nicotinamide-induced type 2 diabetic rats.

Methods

Twenty-four healthy male Wistar rats aged 2 months and weighed 170–200 g were obtained from the Universitas Gadjah Mada Animal Laboratory, Indonesia. Animals were acclimatised for 7 days under standard laboratory conditions (20–25°C, 40–60% humidity, 12-h light/dark cycle) with *ad libitum* food and water, and housed in standard cages (6 rats per cage) with daily bedding replacement. After the adaptation period, the rats were randomly assigned into 4 groups (n=6 each): Healthy, Diabetes, Metformin, and combination of Vitamin D and magnesium (VitDMg) groups.

Induction of Diabetes and Glucose Measurement

Type 2 diabetes mellitus was induced by a single intraperitoneal injection of 45 mg/kg BW streptozotocin (Cayman Chemical, Ann Arbor, MI, USA) dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5), administered 15 min after an intraperitoneal injection of 110 mg/kg BW nicotinamide (Nacalai Tesque, Inc., Kyoto, Japan).(12) To confirm the success of diabetes induction, random blood glucose (RBG) was measured 72 h after injection.(13) Blood was collected from the tail vein (2–3 drops), and RBG levels were determined using the glucose oxidase–peroxidase (GOD-PAP) enzymatic photometric assay (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Rats with RBG levels ≥250 mg/dL were classified as diabetic and included in the experiment, while those below this threshold were excluded.(14) Induction of diabetes was done in all groups, except in the Healthy group.

Animal Treatment Regimens

The treatment was initiated after confirmation of diabetes induction. The Healthy and Diabetes groups received no further pharmacological treatment but were given distilled water via oral gavage to ensure comparable handling. The Metformin group received 9 mg/200 g BW/day metformin (Kalbe Farma, Jakarta, Indonesia) orally. The VitDMg group received a combination of 0.9 IU/200 g BW/day vitamin D (NOW soft gel, NOW Foods, Bloomingdale, IL, USA) and 1.8 mg/200 g BW/day magnesium citrate (Hi-Mg 100 caplet, Imedco, Tangerang, Indonesia) (Figure 1). The doses of vitamin D and magnesium were derived from standard therapeutic doses in humans and converted to equivalent rat doses using the Laurence and Bacharach method.(12) All materials were freshly prepared daily: metformin and magnesium citrate were suspended in distilled water, while vitamin D soft gels were diluted for oral gavage. The gavage volume did not exceed 2 mL/rat/day. Interventions were administered once daily at the same time each morning for 21 consecutive days to minimise circadian variation in metabolic responses.

Ultrasound Doppler Assessment

All rats were put under anaesthesia with 100 mg/kg ketamine hydrochloride (PT. Dexa Medica, Tangerang, Indonesia) intraperitoneally on day-24. Carotid artery tunica intima thickness was measured using a Doppler Logic E Pro device (GE Healthcare, Chicago, IL, USA) equipped with a 12 MHz linear probe. The right chest and neck regions of rats were shaved and coupling gel was applied to optimize imaging. A longitudinal two-dimensional gray-scale scan of the right common carotid artery was obtained with the vessel oriented parallel to the probe surface. The tunica intima was identified as a bright echogenic line along the luminal surface of the arterial wall, and thickness was measured at three distinct points over three consecutive cardiac cycles. The average of these measurements was used as the final value for each animal.(15) All examinations were performed by a senior consultant radiologist with subspecialty training, who was blinded to group allocation, to ensure accuracy and minimize operator-related bias. Inter-observer reliability was not assessed, but all scans were performed by the same radiologist.

Measurement of hs-CRP, IL-12, and Renin Levels

Blood samples were obtained on day-24 from the orbital sinus under anaesthesia, with approximately 3 mL collected from each rat using plain collection tubes. Samples were allowed to clot at room temperature for 2 h and centrifuged at 2,000×g for 20 min to obtain serum. Serum aliquots were prepared and stored at ≤−20 °C until analysis to avoid repeated freeze–thaw cycles. The concentrations of Mouse hs-CRP, Mouse IL-12, and Rat Renin were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA): Mouse hs-CRP ELISA Kit Cat. No. ER1048 (Fine Test, Wuhan, China, with sensitivity 0.094 pg/mL and detection range 0.156–10 pg/mL); Mouse IL-12 ELISA Kit Cat. No. ER1086 (Fine Test with sensitivity 9.375 pg/mL and detection range 15.625–1000 pg/mL); and Rat REN (Renin) ELISA Kit Cat. No. ER0392 (Fine Test with sensitivity 9.375 pg/mL and detection range 15.625-1000pg/mL), respectively. Briefly, 100 µL of standards and samples were added to microplates pre-coated with target-specific capture antibodies and incubated for 90 minutes at 37°C. After removing the liquid and washing the wells twice, 100 µL of biotin-labeled detection antibody was added and incubated for 60 minutes at 37°C. After removing the unbound conjugates, 100 µL of Horseradish Peroxidase (HRP)-conjugated Streptavidin (SABC) was introduced and incubated for 30 minutes at 37°C. The wells were then washed and 90 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and incubated in the dark for 10–20 minutes at 37°C. The colorimetric reaction was terminated by the addition of 50 µL of stop solution, and the optical density (OD) was promptly measured at 450 nm using a microplate reader. The final concentrations were calculated by interpolating the OD values against a four-parameter

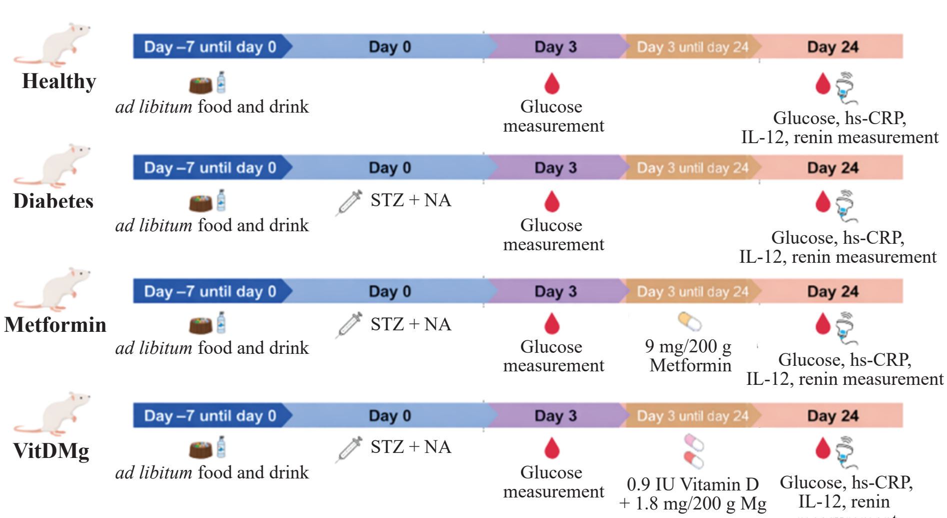

Figure 1. Timeline of the current study and animal treatment based on the assigned groups.

logistic (4-PL) standard curve generated for each specific assay. All assays were performed in duplicate, and intra-assay variability was maintained below 10%.

Results

At baseline (day-0), body weight did not differ significantly among the four groups (p=0.456), confirming that all animals started the experiment under comparable conditions. Meanwhile, RBG levels measured on day-3 showed significant different among the four groups (p<0.05) with the Diabetes, Metformin, and VitDMg groups having FBG levels ≥250 mg/dL, confirming that the diabetes-induction was successful.

Combination of Vitamin D and Magnesium Supplementation Reduced RBG

RBG was also measured before and after the intervention to check the glucose-lowering effect of each therapy. Although RBG in all groups were significantly different between pre-intervention and post-intervention (p<0.05), the highest mean differences were found in VitDMg group which indicated a greater effect in reducing glucose (Figure 2).

Combination of Vitamin D and Magnesium Supplementation Decreased Level of Inflammatory Markers (hs-CRP and IL-12)

The hs-CRP levels increased significantly in the Diabetes group compared to Healthy control (p<0.001), indicating systemic inflammation. Metformin treatment partially reduced hs-CRP, while combination of vitamin D and magnesium supplementation reduced the levels to near-normal values of Healthy group (Figure 3A). IL-12 levels also followed a similar pattern, by being markedly increased in the Diabetes group (p<0.001), and partially reduced with Metformin and combination of vitamin D and magnesium supplementation. But the greatest reduction was found in the VitDMg group (Figure 3B).

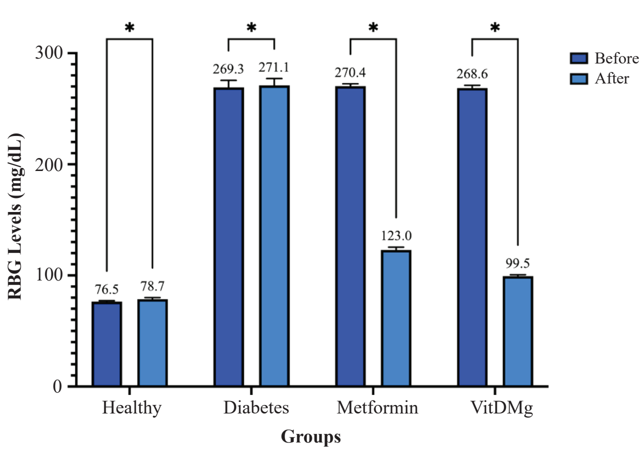

Figure 2. The difference of RBG level between before and after intervention. Values are presented as mean for each group. *indicates significant difference between before and after intervention within each group (p<0.05).

Combination of Vitamin D and Magnesium Supplementation Lowered Renin Level

The highest renin level was found in the Diabetic group. Compared to the Diabetic group, renin decreased almost 40% with vitamin D and magnesium therapy. It was lower than the standard diabetic therapy (metformin). The differences among groups were significant (p<0.001) (Figure 3C).

Combination of Vitamin D and Magnesium Supplementation Thinned Out the Tunica Intima Thickness

The thickness of the tunica intima of the right common carotid artery was significantly greater in the Diabetes group compared with Healthy group (p=0.003). Metformin treatment reduced this thickening, while combination of vitamin D and magnesium supplementation restored intima thickness to values comparable with Healthy controls (Figure 3D, Figure 4).

Discussion

This study demonstrated that the combination of vitamin D and magnesium provided the strongest protective effect by lowering systemic inflammation, affecting renin level, and restoring carotid intima thickness toward normal values. These outcomes highlight its potential as a novel adjunctive strategy in type 2 diabetes mellitus, targeting not only inflammatory pathogenesis but also vascular complications (Figure 5).

The pathophysiology of type 2 diabetes mellitus involves inflammatory processes and tunica intima thickening as in type II DM atherosclerosis. Vitamin D downregulates pro-inflammatory cytokines, suppresses oxidative stress, and enhances NO production, which collectively contribute to improved endothelial function and attenuation of vascular injury.(16,17) On the other hand, magnesium counteracts oxidative stress by inhibiting NADPH oxidase and reducing the generation of ROS (18,19), leading to decreased production of IL-12, CRP, tumor necrosis factor (TNF)-α, and IL-6 (18,20) and its

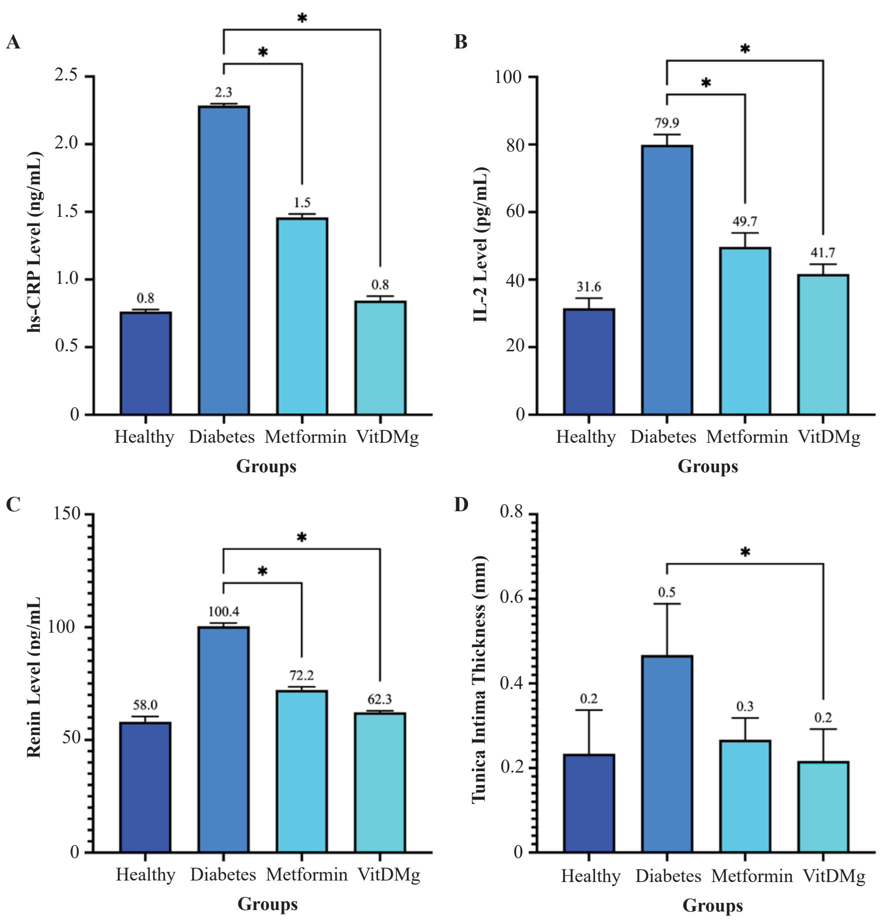

Figure 3. Effects of interventions on inflammatory and vascular markers in the experimental rats. A: hs-CRP expression; B: IL-12 expression; C: renin level; D: tunica intima thickness of the common carotid artery. Values are presented as mean for each group. *indicates significant difference between two groups (p<0.05). Full tables are provided in Supplementary 1.

anti-atherogenic role by increasing NO bioavailability and stabilizing the vascular wall (19,21). These molecular actions correspond with the observed reductions in IL-12 and hs-CRP in the VitDMg group.

The present findings are consistent with previous evidence linking vitamin D deficiency to increased carotid intima-media thickness, while supplementation was shown to preserve vascular integrity.(22) Experimental studies further demonstrated that vitamin D reduced vascular injury and preserved the aortic wall in diabetic rats (23), whereas clinical data confirmed improved carotid remodeling and stiffness in obese adolescents receiving vitamin D supplementation (24). However, pediatric type 1 diabetes data revealed higher common carotid artery intima media thickness (CIMT) without a clear link to vitamin D levels (25), underscoring variability across populations and disease contexts.

In addition to the vascular effect, renin was found elevated in diabetic model. Chronic hyperglycemia also increases the regulation and activity of the enzyme NADPH oxidase, which is the primary trigger for the surge in intracellular ROS production. It interacts synergistically with

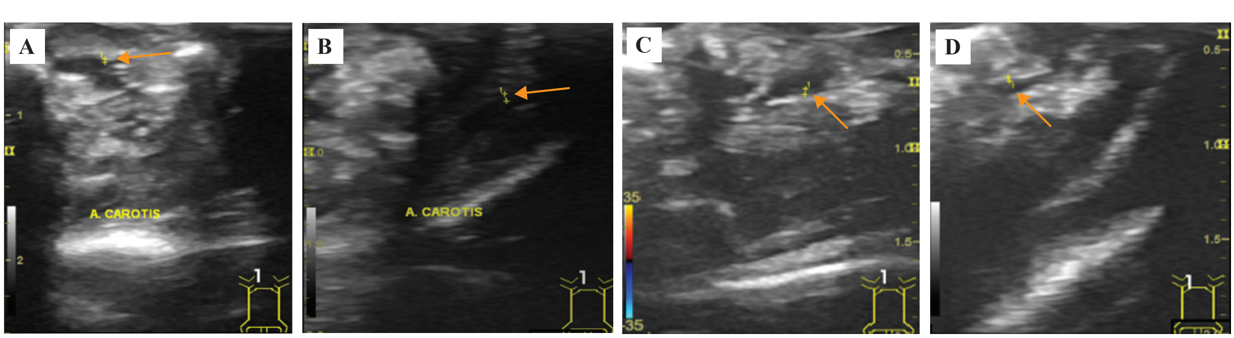

Figure 4. Ultrasound thickness tunica intima of carotid artery following treatment. A: Healthy group; B: Diabetes group; C: Metformin group; D: VitDMg group. Orange arrow: tunica intima.

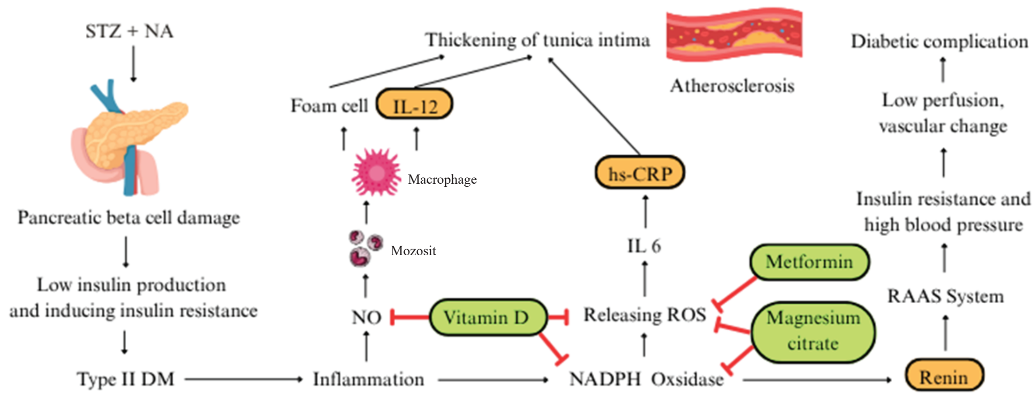

Figure 5. The role of magnesium citrate and Vitamin D in targeting atherosclerosis and diabetic complication. Vitamin D lowered IL-12 level through inhibiting NO production and hs-CRP through ROS retention. Meanwhile, magnesium citrate targeted ROS to decrease hs-CRP level. Both processes suppressed the thickening of tunica intima in atherosclerosis mechanism of type II DM. Vitamin D and Magnesium also inhibited NADPH oxidase to decrease renin which in turn slowed down the complication of type II DM.

metabolic abnormalities to trigger pathological activation of RAAS both systemically and at the tissue level.(26) The enzyme renin increases the production of angiotensin II (Ang-II), which contributes to oxidative stress, insulin resistance, and pancreatic β-cell apoptosis. Furthermore, aldosterone, produced through the RAAS pathway, can inhibit insulin signaling, exacerbate insulin resistance, and disrupt glucose homeostasis.(10) From current research, it was known that the level of renin in diabetic mice with vitamin D and magnesium was far lower than without therapy. It aligned with the previous finding showing that vitamin D supplementation significantly reduced plasma renin level, especially in people with diabetic and coronary artery disease.(27)

Parallel to these observations, magnesium supplementation has also demonstrated vascular benefits. It reduced vascular calcification and improved endothelial function (20), and long-term administration lowered carotid intima-media thickness in patients with diabetes undergoing hemodialysis.(22) A randomized trial confirmed that 24-week magnesium supplementation decreased CIMT, hs-CRP, oxidative stress, and improved metabolic control in diabetic hemodialysis patients.(28) A meta-analysis further supported consistent CIMT reduction, although no significant effect on CRP was detected (29), suggesting that magnesium's vascular benefits may occur independently of systemic inflammation. Consistently, magnesium citrate, when combined with metformin was able to suppress multiple inflammatory mediators in diabetic rats, underscoring its synergistic anti-inflammatory role.(30)

Beyond structural outcomes, inflammatory modulation has also been reported. Higher dietary vitamin D intake was associated with improves FBG level and reduced hs-CRP levels (31), and meta-analyses confirmed significant reductions in circulating CRP following vitamin D supplementation (32). Vitamin D also suppressed IL-12 via nuclear factor-kappaB (NF-κB) inhibition.(33) Nevertheless, other reviews and trials found no consistent anti-inflammatory effects of vitamin D across populations (34,35), highlighting the variability of vitamin D's anti-inflammatory effects.

In contrast, the present findings demonstrate that combined vitamin D and magnesium reduced hs-CRP and IL-12 more effectively than metformin, suggesting that the combination exerts synergistic anti-inflammatory and vasoprotective effects. These findings are consistent with previous experimental evidence showing that combined vitamin D and magnesium supplementation improves glycemic control, insulin resistance, and oxidative stress markers in diabetic models.(36) Another evidence also indicates that magnesium can potentiate metformin's protective actions in diabetic rats (31), supporting its role as a valuable adjuvant partner. Importantly, no prior study has directly evaluated this combination in diabetic models, reinforcing the novelty and translational relevance of the current work. These results suggest that the combination of 0.9 IU vitamin D and 1.8 mg magnesium citrate per 200 gr in mice may serve as an adjunct to standard antidiabetic therapy, complementing the glycaemic effects of metformin by targeting vascular inflammation and early atherosclerotic changes. If confirmed in clinical studies, this strategy could offer a safe, accessible, and cost-effective approach to support endothelial function and delay the progression of diabetic vasculopathy.

This study was conducted in a rat model of type 2 diabetes, which may not fully replicate the complexity of human disease. Studies in higher animal models and clinical trials will be necessary to confirm the translational potential of vitamin D and magnesium combination therapy in preventing diabetes-associated vascular complications. In addition, only a single dose of vitamin D and magnesium citrate was evaluated, so the optimal therapeutic range and safety profile remain uncertain. The duration of the intervention was also relatively short, limiting the ability to assess long-term vascular outcomes. Future research should investigate different dose regimens, extend the duration of supplementation, and include additional biomarkers of vascular function.

Conclusion

This study demonstrated that combined vitamin D and magnesium supplementation decreased hs-CRP, IL-12, renin, and tunica intima thickness. The combination not only improved glycaemic control but also reduced systemic inflammation and preserve vascular changes, with effects comparable or potentially superior to metformin.

Acknowledgments

We'd like to thank Universitas Sebelas Maret (UNS) Surakarta which provide support for publishing this manuscript (PKGR UNS Grant 194.2/UNS27.22/PT.01.03/2024).

Authors Contribution

RFR, FAZ, TSA, RDY, and YY contributed to the conceptualization, methodology, and overall study design. RFR, FAZ, and TSA were responsible for data collection and interpretation, while RFR, FAZ, TSA, and RDY performed the statistical analysis. AF and FGS contributed to data processing, results visualization, manuscript drafting, and critical revision. ATI and SK contributed to data validation and manuscript review. BW, NP, NW, M, TAS, S, and LOW contributed to laboratory analysis, data interpretation, and validation. KYY contributed to clinical supervision and interpretation of vascular findings. All authors contributed to resources, critically reviewed the manuscript, and approved the final version.

Ethical Statement

The protocol of this study was approved by The Health Research Ethics Committee of Dr. Moewardi General Hospital, Indonesia (No. 2.789/XII/HREC/2024).

Conflict of Interest

The authors declared no conflict of interest.

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