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ORIGINAL ARTICLE

Potential cardioprotective effect of Vitamin D and sodium-glucose transport protein 2 inhibitor in improving cardiac hypertrophy and fibrosis in Type 2 diabetic rats

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ABSTRACT

Background: Diabetes mellitus (DM) is a major risk factor for cardiovascular diseases. The progression of myocardial abnormalities due to DM occurs slowly but is progressive and asymptomatic. Sodium-glucose transport protein 2 inhibitors (SGLT-2i) and Vitamin D have potential cardioprotective properties that inhibit cardiomyocyte fibrosis and hypertrophy, which are early structural changes that occur in the heart of DM patients.
Aim: The study aimed to determine the potential protective effects of SGLT-2i and Vitamin D administration on cardiac hypertrophy and fibrosis in Type 2 diabetic rats.
Methods: This is an experimental study with a post-test-only control group design. Thirty-two male Wistar rats were given a high-fat/high-glucose (HF/HG) diet. After 3 weeks, rats were given an injection of streptozotocin (STZ 35 mg/kg) to induce pancreatic damage. The diabetic rats were then divided into four groups (n = 8 per group): untreated diabetic group (HF/HG/STZ), the diabetic group treated with empagliflozin (EMPA) 10 mg/kg body weight (BW) (HF/HG/STZ+EMPA), the diabetic group treated with Vitamin D 225 IU/day (HF/HG/STZ+VitD), and the diabetic group treated with a combination of EMPA 10 mg/kg BW and Vitamin D 225 IU/day (HF/HG/STZ+EMPA+VitD). Treatments were given by oral gavage for 8 weeks. Left ventricular biopsy was performed at week 13 to examine collagen deposition, the cardiomyocyte cross-sectional area (CSA), and the mRNA expression of β-myosin heavy chain (β-MHC) and transforming growth factor-β (TGF-β). All the obtained data were analyzed statistically.
Results: The administration of EMPA, Vitamin D, and combination therapy of EMPA and Vitamin D reduced the mRNA expression of β-MHC and TGF-β in diabetic rats compared to the untreated diabetic group. The administration of EMPA, Vitamin D, and combination therapy also resulted in a decrease in both the cardiomyocyte CSA and collagen deposition. Compared to monotherapy, combination therapy led to significantly better parameter reduction.
Conclusion: Administration of EMPA, Vitamin D, and combination therapy improved cardiac hypertrophy and fibrosis in type 2 diabetic rats.
Relevance for Patients: The combination of Vitamin D and SGLT-2i may be proposed as a cardioprotective strategy and preventive measure to reduce the incidence of cardiovascular disease in patients with Type 2 DM.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide, and diabetes mellitus (DM) is known to be a major risk factor for the progression of cardiovascular diseases [1]. According to the Framingham Heart Study, men and women with DM have a higher risk

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of developing heart failure than people without DM (×2.4 vs. ×5, respectively), regardless of other risk factors (age, heart disease, coronary artery disease, and hypertension) [2]. Cardiomyocyte hypertrophy and cardiac fibrosis are the earliest structural abnormalities in DM, preceding diastolic cardiac dysfunction in heart failure related to DM [3,4]. The expression of cytoskeletal contractile proteins (β-myosin heavy chain [β-MHC]) increases in response to pathogenic stimuli, facilitating the preservation of cardiac contractile function during periods of energy depletion [5]. Apoptosis resulting from hyperglycemia further induces viable cardiomyocytes to undergo pathological hypertrophy as a compensatory mechanism for maintaining cardiac contractile function [4].

Cardiac fibrosis is a process of pathological remodeling and excessive deposition of the extracellular matrix, which causes abnormalities in the composition and quality of the extracellular matrix. The expression of cardiac transforming growth factor beta (TGF-β) has been associated with collagen deposition, myocardial stiffness, and diastolic dysfunction in diabetic rats [6]. Diastolic dysfunction was ameliorated by inhibition of TGF-β in experimental Type 2 DM (T2DM), suggesting a central role of TGF-β signaling in the pathogenesis of heart failure related to DM [7].

Cardiac fibrosis and hypertrophy in T2DM are potentially reversible conditions. The sodium-glucose transport protein 2 inhibitor (SGLT-2i) is an antidiabetic drug that is clinically proven to have cardiovascular benefits, though the underlying pathomechanism is still being studied [8]. Several mechanisms linked to its cardiovascular benefits include the improvement of left ventricular mass and cardiac fibrosis [9]. Similarly, lower Vitamin D levels were identified in the DM population compared to those without DM and were associated with increased HbA1c levels [10,11]. Previous studies indicated that the renin-angiotensin-aldosterone system (RAAS) induces cardiac hypertrophy in Vitamin D receptor (VDR)-knockout mice [12], whereas Vitamin D supplementation reduced renin expression and left ventricular hypertrophy in a hypertensive rat model [13]. Therefore, we aim to determine the possible protective effects of SGLT-2i and Vitamin D administration on cardiac hypertrophy and fibrosis in Type 2 diabetic rats.

2. Materials and Methods

2.1. Animals

This is an experimental study with a post-test-only control group design. Thirty-two male Wistar rats (*Rattus norvegicus*; 10 – 12 weeks; 150 – 200 g) were purchased from the Animal Laboratory of Pharmacology Department, Faculty of Medicine, Udayana University, Indonesia. Each rat was housed in a cage with a lid made of aluminum wire, located indoors with sufficient lighting. Animal studies were conducted according to the regulation by the Institute of Animal Studies Ethics Committee approved by the Faculty of Medicine, Udayana University (ethical clearance #2377/UN14.2.2.VII.14/LT/2022), with all possible measures taken to minimize suffering.

2.2. Materials

Empagliflozin (EMPA) (Jardiance®) 25 mg tablets were purchased from Anugerah Pharmindo Lestari, Indonesia. Each tablet was crushed and dissolved in sterile water and administered at a dose of 10 mg/kg body weight (BW)/day. Liquid cholecalciferol (Vitamin D3) with a concentration of 400 IU/mL (Kid-D®) was purchased from Adiguna Pharmacy, Indonesia, and administered at a dose of 225 IU/day. Streptozotocin (STZ) and all other chemicals and solvents were of analytical grade and procured from Gamma Scientific Biolab, Indonesia.

2.3. Experimental groups

The diabetic rats were divided into four groups (n = 8 per group): untreated diabetic group (high-fat/high-glucose [HF/HG]/STZ), diabetic group treated with EMPA 10 mg/kg BW (HF/HG/STZ+EMPA), diabetic group treated with Vitamin D 225 IU/day (HF/HG/STZ+VitD), and diabetic group treated with a combination of EMPA 10 mg/kg BW and Vitamin D 225 IU/day (HF/HG/STZ+EMPA+VitD). EMPA (Jardiance®) 25 mg tablets were crushed and dissolved in sterile water and given at a dose of 10 mg/kg BW/day. Liquid cholecalciferol (Vitamin D3) with a concentration of 400 IU/mL (Kid-D®) was given at a dose of 225 IU/day. All treatments were given by oral gavage once daily for 8 weeks.

2.4. Experimental procedure

Wistar rats were given a HF/HG diet for 3 weeks, containing 80% normal rat chow, 15% refining lard, and 5% yolk, along with 20% HG drinking water to induce T2DM. Rat chow was supplemented with Vitamin D (800 IU/kg rat chow) to ensure that rats receive Vitamin D according to the recommended daily intake. After 3 weeks of dietary modification, animals were injected with low-dose STZ (35 mg/kg BW) intraperitoneally, prepared by dissolving STZ in a 0.01 M citrate buffer with a pH of 4.5. Fasting blood glucose (FBG) levels were measured 72 h after STZ injection. Increased FBG ≥200 mg/dL was used in experimental studies as the standard in establishing a diabetic rat model [14], and rats with FBG ≥200 mg/dL were included in the study. FBG was measured using Glucometer 4 Accu-Chek® (Roche Diabetes Care, Switzerland). STZ injection can be repeated once with half the initial dose if the blood glucose level has not reached the desired level. Thereafter, the animals were fed an HF/HG diet for an additional 8 weeks. At week 13, the rats were euthanized with ketamine (50 mg/kg BW) and xylazine (10 mg/kg BW), followed by neck dislocation. Subsequently, surgical procedures were performed to extract the heart. The rats were properly buried in accordance with local customs, similar to the burial of a human body. The animal experimental scheme is depicted in Figure 1.

2.5. Histological analysis

A left ventricular biopsy was performed at week 13. The heart tissue samples were fixed with 10% formaldehyde phosphate-buffered solution for 24 h. The fixed tissue was dehydrated, infiltrated, and embedded in liquid paraffin to solidify. The paraffin blocks were sectioned using microtome at a thickness

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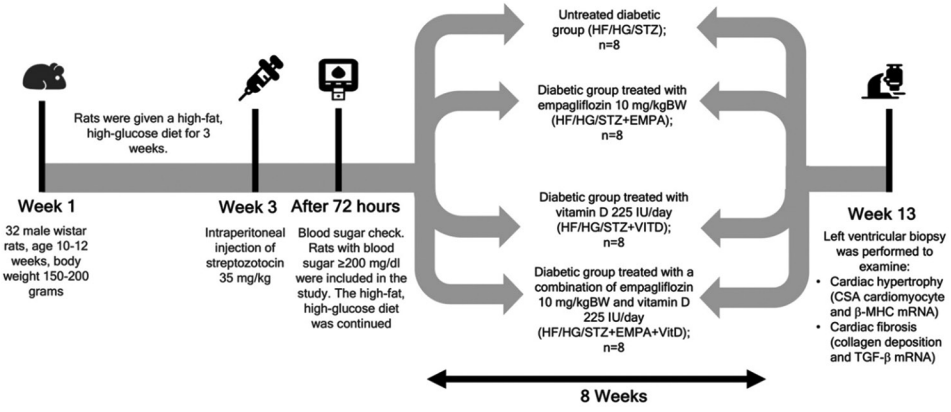

Figure 1. Scheme of animal experiments
Abbreviations: BW: Body weight; HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

of 5 μm and mounted onto a glass slide. The heart samples were histopathologically examined using an Olympus CX40® microscope (Olympus Corporation, Japan) and an Optilab Pro® camera (Miconos, Indonesia). Two different stains, namely hematoxylin and eosin and Picrosirius Red, were used to analyze the cross-sectional area (CSA) of cardiomyocytes and collagen deposition. Each sample was photographed in three visual fields using Optilab Viewer 1.0 software. The cardiomyocyte CSA (μm^2) was measured by averaging the values of the area of five cells for each visual field. Collagen deposition was measured using ImageJ software and quantified in percentage (%). Collagen expression was calculated using the following formula:

$$Collagen\ \exp ression = \frac{Collagen\ pixel\ area}{Total\ tissue\ pixel\ area} \times 100\% \quad (I)$$

2.6. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from heart tissue and preserved with RNA later using the RNeasy Mini Kit (Qiagen). Absolute quantification with one-step qRT-PCR was performed using the KAPA SYBR® FAST One-Step kit (Roche, Switzerland). The concentration of RNA samples was determined using GeneQuant at a wavelength of 260 nm and then diluted to a concentration of 50 ng/μL for each sample. Absolute quantification with one-step qRT-PCR was performed using the following primer sequences: [15,16] β-MHC forward (F): 5'-TCTGGAGGCCTTTGGCAATG-3'; β-MHC reverse (R): 5'-GATGCCAACTTTCCTGTTGC-3'; TGF-β (F): 5′-CAACAATTCCTGGCGTTACCTTGG-3; TGF-β (R): 5′-GAAAGCCCTGTATTCCGTCTCCTT-3′. The amplification was performed in a total volume of 20 μL with the following steps: cDNA synthesis (42°C, 5 min); reverse transcriptase enzyme inactivation (95°C, 5 min); cDNA denaturation (95°C, 3 s); annealing (60°C, 20 s); and 40 cycles. The amplified products were analyzed using 2% agarose gel electrophoresis stained with ×3 Gel Green (Biotium, United States of America [USA]). PCR products were measured using the Dark Reader DR46B Clare Chemical system. Amplification was performed using the MyGo Mini Real-Time PCR thermal cycler (IT-IS Life Science Ltd., United Kingdom) to obtain cycle threshold (Cq) data. A standard curve was derived from purified PCR product and the absolute quantification (fg/μL) was interpolated from Cq and the standard curve.

2.7. Statistical analysis

All the obtained data were analyzed statistically. Data are presented as frequency and mean if distributed normally or as the median if the distribution is non-normal. A one-way analysis of variance (ANOVA) was used for multiple group comparisons, and a least significant difference (LSD) test was used for *post hoc* analysis. $P < 0.05$ was considered statistically significant. The Bliss Independence Model was used to determine the synergistic effect of combination therapy, which is characterized by a measurable effect in the study that is greater than the predicted value of the combined effect.

3. Results

3.1. Baseline characteristics

There were no significant differences in baseline and post-diabetic induction BW or blood glucose levels among the experimental groups, ensuring comparability of the groups' characteristics before treatment initiation (Table 1). We observed an increase in BW in all groups following a 3-week administration of an HF diet and diabetic induction, relative to baseline measurements. At the end of the study, the entire group exhibited a reduction in BW in contrast to their BW during the diabetes induction phase; however, no statistically significant differences in BW were observed among the groups (Table 1). All three treatment groups also had significant reductions in blood glucose after 8 weeks of treatment, relative to the untreated diabetic group (Table 2).

3.2. Effect of EMPA and Vitamin D on the expression of β-MHC mRNA

Comparative tests using one-way ANOVA displayed a significant difference in the mRNA expression of β-MHC

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Table 1. Characteristics of the experimental groups at baseline, following diabetic induction, and post-treatment administration

Characteristic	Experimental groups				P^a
	HF/HG/STZ	HF/HG/STZ+EMPA	HF/HG/STZ+VitD	HF/HG/STZ+EMPA+VitD	
Weight (g)					
Baseline	159.37±22.58	162.50±30.58	155.62±31.67	151.42±11.07	0.854
Post-diabetic induction	187.37±26.74	203.25±32.99	189.00±23.73	179.00±18.40	0.358
Post-treatment	183.00±21.43	169.37±13.90	173.62±33.29	158.71±33.29	0.250
Fasting blood glucose (mg/dL)					
Baseline	110.00±7.69	115.00±8.00	109.37±6.47	110.00±4.32	0.343
Post-diabetic induction	422.12±180.79	524.37±109.26	437.25±206.71	390.00±54.09	0.370
Post-treatment	376.12±117.66	150.87±29.29	160.25±56.77	147.42±60.73	<0.001*

Note: [a]*P*-values were obtained using one-way analysis of variance to compare the differences in mean among groups; *P<0.05.
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D.

Table 2. *Post-hoc* analyses of fasting blood glucose levels between experimental groups

Group	Difference in blood glucose levels, mean±SD (mg/dL)			
	HF/HG/STZ	HF/HG/STZ+EMPA	HF/HG/STZ+VitD	HF/HG/STZ+EMPA+VitD
HF/HG/STZ	-	225.25±36.97 (P<0.001)	215.87±36.97 (P<0.001)	228.69±38.26 (P<0.001)
HF/HG/STZ+EMPA	−225.25±36.97 (P<0.001)	-	−9.37±36.97 (P=0.802)	3.44±38.26 (P=0.929)
HF/HG/STZ+VitD	−215.87±36.97 (P<0.001)	9.37±36.97 (P=0.802)	-	12.82±38.26 (P=0.740)
HF/HG/STZ+EMPA+VitD	−228.69±38.26 (P<0.001)	−3.44±38.26 (P=0.929)	−12.82±38.26 (P=0.740)	-

Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D.

among the four groups (P < 0.001) (Figure 2). Differences in the expression of β-MHC mRNA between each group were then analyzed using LSD analysis. The administration of EMPA (HF/HG/STZ+EMPA group) significantly decreased β-MHC mRNA expression compared to the untreated diabetic group, which received only an HF/HG diet (mean difference: 30.04 fg/μL; 95% confidence interval [CI]: 7.73 – 52.36; P = 0.010). The β-MHC mRNA expression was also significantly lower in the HF/HG/STZ+VitD group compared to the untreated diabetic group (mean difference: 56.16 fg/μL; 95% CI: 33.85 – 78.48; P < 0.001). The highest reduction in mRNA β-MHC expression was observed in the HF/HG/STZ+EMPA+VitD group compared to the untreated diabetic group (mean difference: 80.49 fg/μL; 95% CI: 57.39 – 103.59; P < 0.001). Compared to the HF/HG/STZ+EMPA group, administration of Vitamin D demonstrated a better reduction in mRNA β-MHC expression (mean difference: 26.12 fg/μL; 95% CI: 3.81 – 48.43; P = 0.023). Combination therapy of EMPA and Vitamin D also provided a better reduction in mRNA β-MHC expression compared to monotherapy with EMPA (mean difference: 50.45 fg/μL; 95% CI: 27.35 – 73.54; P < 0.001) or Vitamin D (mean difference: 24.32 fg/μL; 95% CI: 1.22 – 47.42; P < 0.040). The Bliss Independence Model assessed whether the drug combination had a synergistic effect compared to single therapy (Figure 3). Assuming the expression of β-MHC mRNA in the untreated diabetic group was 100%, the expression of β-MHC mRNA was 69.3% in the HF/HG/STZ+EMPA group and 42.6% in the HF/HG/STZ+VitD group. Therefore, the predicted

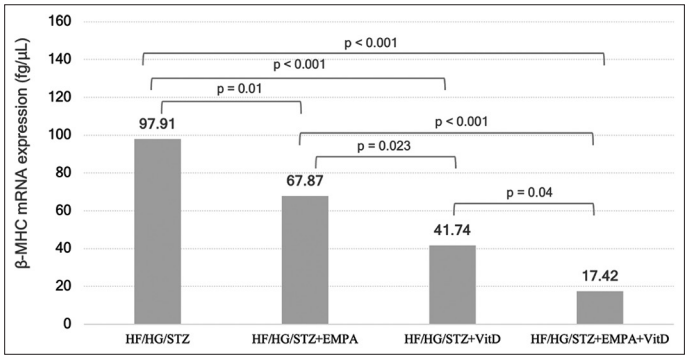

Figure 2. Effect of EMPA, VitD, and combination therapy on β-MHC mRNA expression
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; β-MHC: β-myosin heavy chain

combination response of the HF/HG/STZ+EMPA+VitD group was calculated to be 29.55% (69.3% × 42.6%). In this study, the observed combination response in the HF/HG/STZ+EMPA+VitD group was 17.79%, indicating that the combination therapy of EMPA and Vitamin D has a synergistic effect in reducing the expression of β-MHC mRNA.

3.3. Effect of EMPA and Vitamin D on the cardiomyocyte CSA

Comparative analysis using one-way ANOVA displayed a significant difference in cardiomyocyte CSA among the four groups (P < 0.001) (Figure 4). Compared to the untreated diabetic group, the highest reduction of cardiomyocyte CSA was obtained

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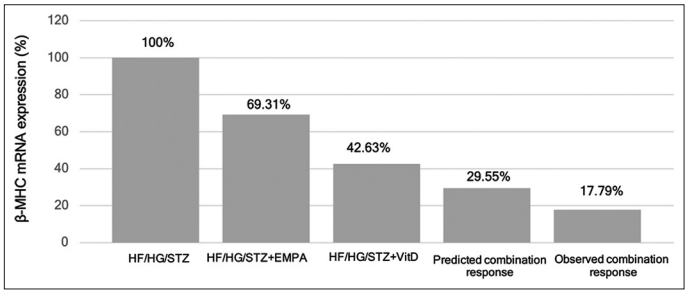

Figure 3. Synergistic effect of combination therapy on reducing β-MHC mRNA expression
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; β-MHC: β-myosin heavy chain

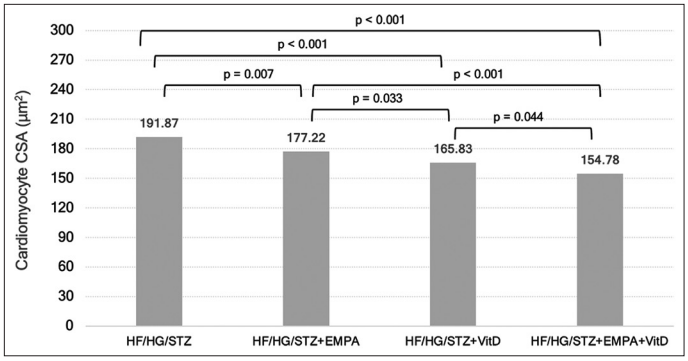

Figure 4. Effect of EMPA, VitD, and combination therapy on cardiomyocyte CSA
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; CSA: Cross-sectional area

in the HF/HG/STZ+EMPA+VitD group (mean difference: 37.08 μm²; 95% CI: 26.35 – 47.81; $P < 0.001$). Monotherapy with EMPA or Vitamin D also significantly reduced cardiomyocyte CSA compared to the untreated diabetic group, with mean differences of 14.65 μm² (95% CI: 4.28 – 25.01; $P = 0.007$) and 26.03 μm² (95% CI: 15.67 – 36.40; $P < 0.001$), respectively. Compared to the HF/HG/STZ+EMPA group, Vitamin D administration demonstrated a higher reduction in cardiomyocyte CSA (mean difference: 11.38 μm²; 95% CI: 1.02 – 21.75; $P = 0.033$). Combination therapy of EMPA and Vitamin D also provided a greater reduction of cardiomyocyte CSA compared to monotherapy using EMPA (mean difference: 22.43 μm²; 95% CI: 11.70 – 33.16; $P < 0.001$) or Vitamin D (mean difference: 11.05 μm²; 95% CI: 0.32 – 21.78; $P = 0.044$) (Figure 5). Using the Bliss Independence Model, the predicted combination response of the HF/HG/STZ+EMPA+VitD group on cardiomyocyte CSA was 79.81% (Figure 6). The observed combination response of the HF/HG/STZ+EMPA+VitD group in this study was 80.67%, indicating that the combination therapy of EMPA and Vitamin D is additive but not synergistic in reducing cardiomyocyte CSA.

3.4. Effect of EMPA and Vitamin D on the expression of TGF-β mRNA

We observed gradual decrease in TGF-β mRNA expression following administration of EMPA (mean difference: 13.78 fg/μL; 95% CI: 1.09 – 28.66; $P = 0.048$), Vitamin D (mean difference: 26.69 fg/μL; 95% CI: 11.82 – 41.57; $P = 0.001$), and combination therapy (mean difference: 43.43 fg/μL; 95% CI: 28.03 – 58.83; $P < 0.001$) compared to the untreated diabetic group (Figure 7). The HF/HG/STZ+VitD group tended to express lower levels of TGF-β mRNA compared to the HF/HG/STZ+EMPA group, though not statistically significant (mean difference: 12.91 fg/μL; 95% CI: −1.96 – 27.79; $P = 0.086$). Compared to monotherapy, combination therapy displayed the greatest reduction of TGF-β mRNA expression. Based on the Bliss Independence Model, the predicted combination response of the HF/HG/STZ+EMPA+VitD group on TGF-β mRNA expression was 48.2%, while the observed combination response was 43.4% (Figure 8). This indicates that the administration of combination therapy has a synergistic effect on the reduction of TGF-β mRNA expression.

3.5. Effect of EMPA and Vitamin D on collagen deposition

Based on its ability to reduce collagen deposition, the administration of combination therapy (mean difference: 9.41%; 95% CI: 6.87 – 11.96; $P < 0.001$) led to a significantly greater parameter reduction compared to EMPA (mean difference: 2.65%; 95% CI: 0.19 – 5.11; $P = 0.035$) and Vitamin D (mean difference: 6.19%; 95% CI: 3.74 – 8.65; $P < 0.001$) monotherapy (Figure 9). When comparing the effectiveness of both monotherapies, we found that Vitamin D led to a significantly greater reduction of collagen deposition than EMPA (mean difference: 3.54%; 95% CI: 1.08 – 6.00; $P = 0.006$) (Figure 10). Using the Bliss Independence Model, combination therapy was observed to have a synergistic effect on the reduction of collagen deposition, with a lower percentage of collagen deposition in the observed combination response (41.8%) compared to the predicted combination response (51.6%) (Figure 11).

4. Discussion

Both T2DM and hyperglycemia are pathological hypertrophic stimuli in cardiomyocytes. At the cellular level, cardiac hypertrophy refers to an increase in the size of cardiomyocytes accompanied by elevated protein synthesis and structural changes in sarcomeres [17]. This study uses two quantitative parameters to assess cardiac hypertrophy, namely, the cardiomyocyte CSA and the mRNA expression of the contractile cytoskeletal β-MHC protein as a marker of hypertrophy [17]. Apoptosis, resulting from hyperglycemia-induced stress, causes viable cardiomyocytes to undergo hypertrophy to compensate for cardiac pump function [4,5]. Exposure to pathological stimuli increases the expression of the contractile cytoskeletal β-MHC protein, maintaining cardiomyocyte contractility under energy-deficient conditions. As cell viscosity reaches a critical threshold, cardiomyocytes enlarge and can be quantified histopathologically based on the cardiomyocyte CSA [5].

Hyperglycemia, hyperinsulinemia, and insulin resistance in T2DM are also known to induce pathological remodeling and excessive deposition of the extracellular matrix [18]. Excessive deposition of the extracellular matrix, including

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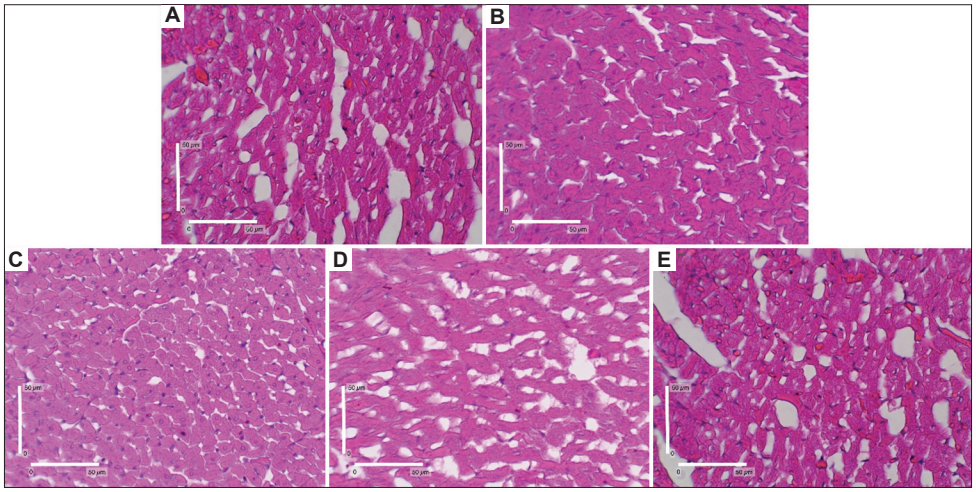

Figure 5. Haematoxylin and eosin staining of the cross-sectional tissue slices of the rat's left ventricle: (A) normal rat tissue; (B) HF/HG/STZ; (C) HF/HG/STZ+EMPA; (D) HF/HG/STZ+VitD; and (E) HF/HG/STZ+EMPA+VitD. Scale bars: 50 μm. Magnification: ×400
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

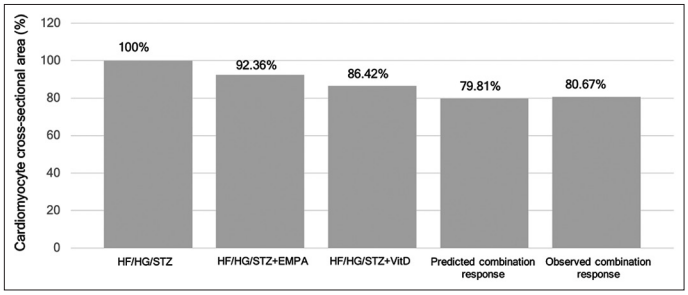

Figure 6. Additive effect of combination therapy on reducing cardiomyocyte cross-sectional area
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

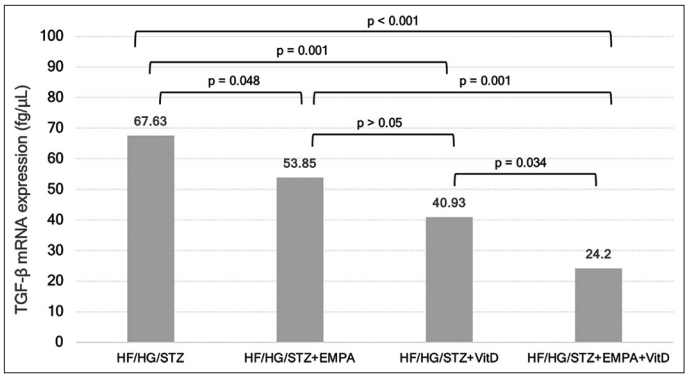

Figure 7. Effect of EMPA, VitD, and combination therapy on TGF-β mRNA expression
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; TGF-β: Transforming growth factor-β

Type I and III collagen, is a characteristic of cardiac fibrosis in T2DM. Similarly, the expression of cardiac TGF-β is associated with collagen deposition, myocardial stiffness, and diastolic dysfunction in diabetic rats [6]. Previous findings demonstrated that diastolic dysfunction was ameliorated by inhibition of TGF-β

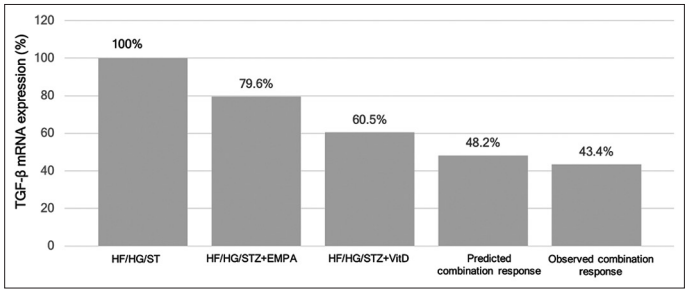

Figure 8. Synergistic effect of combination therapy on reducing TGF-β mRNA expression
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; TGF-β: Transforming growth factor-β

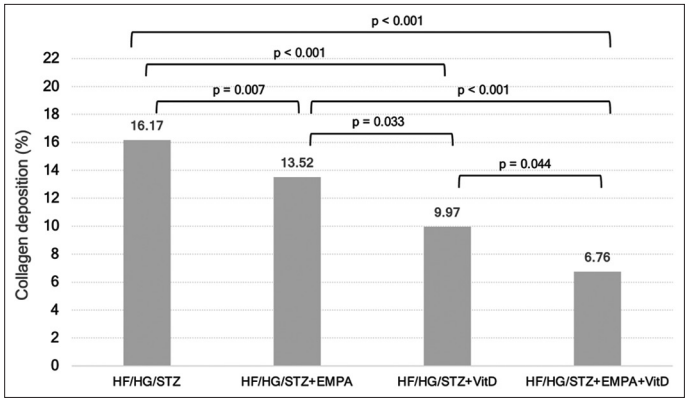

Figure 9. Effect of EMPA, VitD, and combination therapy on collagen deposition
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

in experimental T2DM, suggesting a prominent role of TGF-β signaling in the pathogenesis of DM-related heart failure [7]. Therefore, we measured the expression of TGF-β mRNA and collagen deposition to quantitatively assess cardiac fibrosis.

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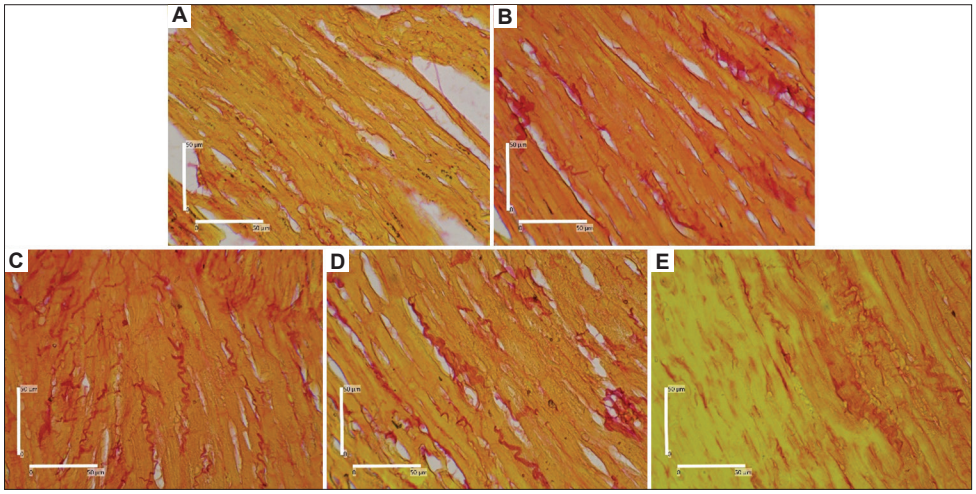

Figure 10. Picrosirius Red staining of collagen (red) in the cross-sectional tissue slices of the rat's left ventricle: (A) normal rat tissue; (B) HF/HG/STZ; (C) HF/HG/STZ+EMPA; (D) HF/HG/STZ+VitD; and (E) HF/HG/STZ+EMPA+VitD. Scale bars: 50 μm. Magnification: ×400
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

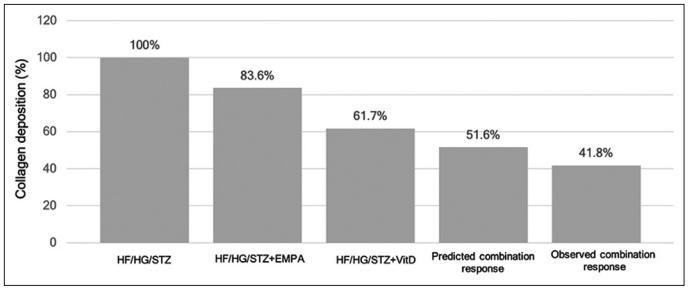

Figure 11. Synergistic effect of combination therapy on reducing collagen deposition
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

Our study indicated that administration of the SGLT-2i EMPA resulted in a significant reduction of cardiac hypertrophy and fibrosis parameters in T2DM rats compared to the untreated diabetic rats. Our findings are consistent with previous studies, where the administration of EMPA significantly reduces β-MHC mRNA expression, TGF-β mRNA expression, cardiomyocyte size, and collagen deposition in myocardial infarction and diabetic rat models [15,19-21]. The cardioprotective effects of EMPA have been described in previous studies, which suggest increased SERCA2a/PLN expression ratio, inhibition of NHE1 activity, improved myocardial energetics, decreased NLRP3 inflammasome, decreased oxidative stress, direct inhibition of TGF-β, and activation of sirtuins as the potential mechanisms [15,19,20,22,23].

Sodium-glucose transport protein 2 (SGLT-2) channels are not expressed in cardiomyocytes, but it is known that SGLT-2i influences Ca^{2+} homeostasis by modulating Na^{+} in the cytoplasm of cardiomyocytes. Calcium ion (Ca^{2+}) is essentially involved in excitation-contraction coupling and also serves as a second messenger that regulates the transcription of genes related to cardiac hypertrophy and other maladaptive remodeling pathways [24]. EMPA works by regulating Ca^{2+} homeostasis, achieved by enhancing the expression ratio of SERCA2a/PLN and suppressing NHE1 activity [19,24]. An increase in the SERCA2a/PLN expression ratio will lead to an increase in Ca^{2+} mobilization toward the endoplasmic reticulum, whereas the inhibition of NHE1 lowers intracellular Na^{+} levels and facilitates the extracellular release of Ca^{2+} through the NCX pump. Both mechanisms effectively prevent intracellular Ca^{2+} accumulation which could trigger cardiac remodeling [25]. The elevation of β-hydroxybutyrate following EMPA administration serves as a more efficient alternative substrate that improves myocardial energetics [15,24]. β-hydroxybutyrate is also recognized as a contributing factor to the reduction of NLRP3 inflammasome, which, in turn, is associated with the presence of chronic inflammation in patients with heart failure [26,27] Nutrient deprivation state secondary to glycosuria from EMPA administration also triggered the activation of proteins known as sirtuins (Sirt1, Sirt3, Sirt6) [23]. The activation of Sirt1 is not only beneficial for inducing autophagy of dysfunctional organelles, but also contributes to the reduction of cardiac fibrosis induced by TGF-β [23].

The VDR and 1-α-hydroxylase enzyme, which converts Vitamin D to its active form, are both found in cardiovascular tissue. Vitamin D deficiency triggers cardiac hypertrophy and activation of the fetal gene program (increased β-MHC expression), which is also observed in failing hearts [28,29]. Aligned with our findings, previous studies suggest that Vitamin D supplementation significantly reduces the expression of β-MHC mRNA, TGF-β, cardiomyocyte CSA, and fibrosis in hypertrophic rat models induced by pressure overload and uremia [30,31]. This parameter reduction is attributed to increased SERCA2a, decreased fibroblast growth factor-23 (FGF23) expression [30,31], inhibition of NF-κB activation [32], decreased renin and oxidative stress levels [13,33], and inhibition of TGF-β/Smad pathway [34]. *In vitro* studies also suggest that supplementation of $1\alpha,25(OH)_2D_3$ plays a role in reducing β-MHC expression directly, as demonstrated by its

ability to suppress β-MHC expression in wild-type rats without Vitamin D deficiency [35].

Our study demonstrated a novel investigation into the combined administration of Vitamin D and EMPA, revealing enhanced antihypertrophic and antifibrotic effects on the myocardium of diabetic rats, an area that has not been previously explored. These synergistic effects might occur because each monotherapy targets different mechanisms of action in reducing cardiac fibrosis and hypertrophy. Furthermore, lower Vitamin D levels were observed in the DM population compared to those without it and were associated with increased HbA1c levels [11]. It is hypothesized that the accumulation of Vitamin D in the adipose tissue of patients with T2DM reduces its availability in circulation, whereas Vitamin D is required for facilitating gene transcription and insulin exocytosis [36,37].

EMPA treatment in patients with T2DM has been reported to transiently increase FGF-23 and decrease 1,25-dihydroxy Vitamin D levels [38]. This observation may reflect a temporary increase in sodium-driven phosphate reabsorption in the proximal tubule of the kidney in response to SGLT-2 inhibition [38] After initiating the SGLT-2i, changes in fluid status have also been observed, which is accompanied by elevated plasma renin activity and serum aldosterone concentration after 30 days, suggesting increased RAAS activity, with normalization after 6 months [39]. The administration of Vitamin D was previously reported to reduce renin levels and inhibit FGF-23, both of which contribute to cardiac hypertrophy and fibrosis [30]. Hence, we hypothesize that the combination of Vitamin D with SGLT-2i administration will yield improved outcomes.

Our study concluded that the administration of EMPA, Vitamin D, and combination therapy of EMPA and Vitamin D significantly reduced the expression of cardiac fibrosis and hypertrophy compared to untreated diabetic groups. However, a significant reduction in parameters of cardiac hypertrophy and fibrosis was observed (but not to the normal healthy baseline) in comparison to the untreated diabetic group. Thus, while the treatments demonstrated efficacy in reducing these parameters relative to the diabetic control, it remains uncertain whether the levels achieved are comparable to those in healthy subjects. Further research is warranted to determine if the treatments can restore cardiac health to normal levels.

We did not objectively measure the appetite, water intake, urine output, and blood pressure of our experimental animal. Hence, we were unable to evaluate the diuretic and blood pressure-lowering effect of SGLT-2i and Vitamin D. Our study did not examine Vitamin D levels in rats before and after induction of DM, and after administration of treatment. Therefore, it cannot be concluded whether T2DM impacts Vitamin D blood levels and leads to secondary Vitamin D deficiency as the disease advances.

Our study also did not assess cardiac function due to the limitation of our animal laboratory to conduct proper echocardiographic procedures and measurements. Consequently, we are unable to determine whether the improvements in structural changes correspond to functional cardiac improvements. In addition, we did not include normal healthy controls in this study. Hence, we are unable to compare the obtained reverse remodeling effects with those of a normal control.

This study serves as a pilot study, establishing the groundwork for future studies that will focus on specific pathways or the activation of proteins that were not investigated in this study, namely SERCA2a/PLN, NHE1, β-hydroxybutyrate, NLRP3 inflammasome, and sirtuins. These pathways are speculated to drive the mechanism responsible for the observed cardioprotective effect resulting from the combined therapy of SGLT-2i and Vitamin D.

5. Conclusion

Administration of EMPA, Vitamin D, and combination therapy improved cardiac hypertrophy and fibrosis in T2DM rats. Compared to monotherapy, the combination therapy of EMPA and Vitamin D led to significantly better parameter reductions.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethics Approval and Consent to Participate

This research was approved by the Ethics Commission of the Faculty of Medicine, Udayana University (approval number: 2395/UN14.2.2.VII.14/LT/2022).

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author on reasonable request.

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