Ketogenic diet regulates cardiac remodeling and calcium homeostasis in diabetic cardiomyopathy

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Abstract

Background

Ketogenic diet (KD) might alleviate patients with diabetic cardiomyopathy. However, the underlying mechanism remains unclear. Myocardial function and arrhythmogenesis depend on the homeostasis of calcium (Ca$^{2+}$). We investigated the effects of KD on Ca$^{2+}$ homeostasis and electrophysiology in diabetic cardiomyopathy.

Methods

Male Wistar rats were created to have diabetes mellitus (DM) by applying streptozotocin (65 mg/kg intraperitoneally), and they were treated for 6 weeks with a normal diet (ND) or KD. Our electrophysiological and Western blot analyses assessed myocardial Ca$^{2+}$ homeostasis in ventricular preparations in vivo.

Results

Unlike those on KD, DM rats treated with ND exhibited a prolonged QTc interval and action potential duration. Compared to control and DM rats on KD, DM rats treated with ND also showed lower intracellular Ca$^{2+}$ transients, sarcoplasmic reticular Ca$^{2+}$ content, sodium (Na$^+$)-Ca$^{2+}$ exchanger currents (reverse mode), L-type Ca$^{2+}$ contents, sarcoplasmic reticulum ATPase contents, Cav1.2 contents, phosphorylated phospholamban/phospholamban ratios, and phosphorylated ryanodine receptor 2 (RyR2) at serine 2808/RyR2 ratios but higher Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII-δ) levels. Moreover, DM rats treated with ND demonstrated a higher frequency and incidence of Ca$^{2+}$ leak, mitochondrial and cytosolic reactive oxygen species, Na$^+$/hydrogen-exchanger currents, and late Na$^+$ currents than control and DM rats on KD.

Conclusion

KD treatment may attenuate the effects of DM-dysregulated Na$^+$ and Ca$^{2+}$ homeostasis, contributing to its cardioprotection in DM.

Introduction

Low-carbohydrate diets are gaining popularity recently because of the increased awareness regarding the harmful metabolic effects of processed carbohydrates. The ketogenic diet (KD) is a high-fat, low-carbohydrate diet that serves around 60% of fats for daily energy needs. It has been linked to mitigating epileptic seizures in children[1] and several other neurological conditions[2]. When dietary fat replaces
carbohydrates in the liver, fat will be transformed into ketones, including beta-hydroxybutyrate (BHB), acetoacetate, acetone, and fatty acids (FAs). Evidence of KD’s possible advantages to health includes neurological illnesses, cancer, and mitochondrial diseases [3–5]. BHB improved endogenous antioxidant defense and mitochondrial performance [5]. Moreover, increased circulating ketone levels in patients with heart failure may also improve their cardiac health [4, 6] because, as oxygen-efficient, highly energetic fuel, ketone bodies may enhance the work capacity of the myocardium at risk [7, 8].

Patients with diabetes mellitus (DM) may have cardiomyopathy after a while [9], possibly due to mitochondrial dysfunction and calcium (Ca²⁺) dysregulation [10]. The pathological impact of diabetic cardiomyopathy in terms of its underlying mechanism and functional implications warrants clarification; as such, the optimal therapeutic strategy for diabetic cardiomyopathy remains unknown. Moreover, whether KD will modulate sodium (Na⁺) or Ca²⁺ homeostasis through its potential consequences on the myocardial electrical and structural remodeling in DM hearts remains unclear. Our earlier investigation demonstrated that sodium-glucose co-transporter-2 inhibitor (SLGT2i) modulated Na⁺ or Ca²⁺ homeostasis by decreasing oxidative stress in diabetic cardiomyopathy [11]. In the cardiomyocytes, SLGT2i may increase energy utilization from the ketone bodies, thereby improving the myocardial function with an antiarrhythmic potential [12]. Furthermore, we recently also illustrated that empagliflozin regulates glucose and FA metabolism [13]. Consequently, we postulate that KD may influence the electrical and structural remodeling of the heart and alters the Na⁺ or Ca²⁺ homeostasis in patients with diabetic cardiomyopathy.

Material and Methods

The Supplementary material online reports more thorough procedures.

Animal experiments

As shown in Fig. 1, the Institutional Animal Care and Use Committee Panel of Taipei Medical University reviewed and approved our animal experimentation protocols with the IACUC/IACUP approval number LAC 2020 – 0478. The Guide for the Care and Use of Laboratory Animals (eighth edition), published by the US National Academies Press (NBK54050) in 2011, was also followed. All animal works were conducted in the TMU Laboratory Animal Center located at the Taipei Medical University. Wistar male rats at 8 weeks of age were maintained in a controlled setting with a temperature of 21.2°C, a 12-hour light-dark cycle with unlimited commercial rat food and water. Similar to our previous investigation [14], control rats and DM rats on KD had higher heart weight, lower heart-to-body weight ratio, and better-left ventricle (LV) function than DM rats on ND did (Supplementary Table 1 and Fig. 1). Electrocardiography was performed at the age of 16 weeks. Rats were anesthetized by inhalation using 2.0–2.5% isoflurane (5% in oxygen; Panion & BF biotech, Taoyuan, Taiwan). Their hemodynamics were stabilized after 30 min under continuous electrocardiography monitoring. The electrocardiogram tracings were collected using Bio-amplifier’s standard lead II limb with an ML 845 Powerlab polygraph recorder AD Instruments (Castle
Hill, Australia) [15]. The readings of control and DM rats on KD and normal diet (ND) were recorded continually.

The male Wistar rats were initially sedated with zoletil (10 mg/kg; Virbac, Carros, France) and xylazine (5 mg/kg; Bayer, Leverkusen, Germany) intramuscularly. Then, they were terminated with an overdose of isoflurane inhalation with a precision vaporizer (5% in oxygen; Panion & BF biotech, Taoyuan, Taiwan) [11, 16]. The dosage of anesthesia was confirmed to be adequate based on the absence of chest movement, palpable heartbeat, corneal reflexes, and no response to toe pinch. The hearts were removed through midline thoracotomy for further electrophysiological, confocal microscopy, and Western blot experiments.

Measurement of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)), sarcoplasmic reticulum (SR) Ca\(^{2+}\) transient, and Ca\(^{2+}\) store

Ventricular myocytes isolated from the rats were enzymatically separated using type XIV protease (Sigma) and type I collagenase (Sigma). As described previously[17], the ventricular myocytes were retrogradely perfused with digestive enzymes, and based on the fluorometric ratio (fluo-3 fluorescence), the intracellular Ca\(^{2+}\) [Ca\(^{2+}\)]\(_{i}\) was calculated. In brief, the cells at room temperature were treated with a fluorescent Ca\(^{2+}\) (10 µM) fluo-3/AM for 30 min. Next, the bath solution was changed, and fluo-3/AM was allowed to undergo intracellular hydrolysis to remove the extracellular dye. At > 515 nm, emission was confirmed with an argon-ion laser 3 fluorescence stimulated at 488 nm. The cells were repeatedly scanned at 2-ms intervals using a line scan (8-bit) imaging. Fluorescent cells were monitored using Zeiss LSM 510 (Carl Zeiss, Jena, Germany) laser scanning confocal microscope and an Axiovert 100 inverted microscope (Carl Zeiss, Jena, Germany). The transient [Ca\(^{2+}\)]\(_{i}\) changes were calculated by measuring the fluorescent signals and baseline values (F/F\(_{0}\)). The fluorescence of fluorescent signals (F) was compared to that of the baseline fluorescence (F\(_{0}\)), variations in dye concentrations were corrected, and fluorescence intensity variations generated by various dye injection volumes were excluded [18]. [Ca\(^{2+}\)]\(_{i}\) transients, diastolic [Ca\(^{2+}\)]\(_{i}\), peak systolic [Ca\(^{2+}\)]\(_{i}\), and decaying fraction [Ca\(^{2+}\)]\(_{i}\) transients were monitored using 10-ms twice-threshold strength square-wave pulses after 1-Hz field stimulation. Moreover, the monoexponential least-squares fit was used to calculate [Ca\(^{2+}\)]\(_{i}\). After a steady-state [Ca\(^{2+}\)]\(_{i}\) transient was achieved with multiple pulses, the superfusate containing tetracaine (1mmol/L) for the 20s was changed to 0 Na\(^{+}\)/0 Ca\(^{2+}\) solutions (1 Hz for 15 s). The 1 mmol/L tetracaine-reduced [Ca\(^{2+}\)]\(_{i}\) was used to quantifying the SR Ca\(^{2+}\) leak as previously described [19].

Fast determination of SR Ca\(^{2+}\) reserves was measured using a 30-s pulse stimulation train at 1 Hz followed by the addition of 20 mmol/L caffeine. The SR Ca\(^{2+}\) reserve was calculated from the peak amplitude of the caffeine-induced Ca\(^{2+}\) transient. 20 mmol/L of caffeine voltage-clamped at −40 mV was quickly injected into cells. As previously mentioned [20–22], the SR Ca\(^{2+}\) level was calculated using the integral of the inward Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) current as follows:

\[
\text{SR Ca}^{2+} \text{ content (mol/L/L cytosol) = } \frac{[(1 + 0.12 \text{Ccaff/F} \times 1000)]}{(Cm \times 8.31 \times 8.44)}.
\]
Besides the cell surface-to-volume ratio being 8.44 pF/pL, Ccaff is the integral of the inward NCX current caused by caffeine, F is Faraday's number, and Cm is the capacitance of the membrane.

**AP and ionic currents**

Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) at 35 ± 1°C was used on the newly isolated rat ventricular myocytes to perform whole-cell patch-clamp. In the voltage-clamp mode, ionic currents were reported, and action potentials (APs) directed at 1 Hz in the current-clamp mode were identified. L-type Ca$^{2+}$ current ($I_{\text{Ca-L}}$) was measured using micropipettes filled with a solution composed of 130 mM CsCl, 1 mM MgCl$_2$, 5 mM MgATP, 10 mM HEPES, 5 mM Na$^{2+}$ phosphocreatine, 10 mM EGTA, and 0.1 mM NaGTP. The NCX current was measured with 110 mM CsCl, 20 mM NaCl, 1.75 mM CaCl$_2$, 20 mM tetraethylammonium, 0.4 mM MgCl$_2$, 10 mM HEPES, 5 mM 1,2-bis(2-amino phenoxy) glucose, 75 mM 1,2-bis(2-amino phenoxy) ethane-N,N,N',N'-tetraacetic acid, and 5 mM MgATP titrated to a pH of 7.25. The late sodium current ($I_{\text{Na-Late}}$) was measured using 130 mM CsCl, 10 mM NaCl, 5 mM HEPES, 5 mM EGTA, 5 mM MgATP, and 5 mM glucose. The action potential (AP) was measured using 110 mM K aspartate, 20 mM KCl, 5 mM MgATP, 1 mM MgCl$_2$, 0.5 mM EGTA, 10 mM HEPES, 5 mM Na$^{2+}$ phosphocreatine, and 0.1 mM LiGTP titrated with KOH to a pH of 7.2. A digital-to-analog converter (12-bit) was used to create voltage command pulses on pCLAMP (Axon Instruments). A low-pass filtration was applied to the recordings during half the sampling frequency. The AP amplitude (APA) was assessed as the difference between the peak AP depolarization and resting membrane potential (RMP). The repolarization of AP durations (APDs) at 90% (APD$_{90}$), 50% (APD$_{50}$), and 20% (APD$_{20}$) were calculated.

$I_{\text{Ca-L}}$ was measured during depolarization from a holding potential of −50 mV at 0.1 Hz as an inward current to evaluate the possibilities of −40 to +60 mV in 10-mV increments for 300 ms. CsCl and tetraethylammonium chloride instead of NaCl and KCl were used along with the external solution. We used pulses between −100 and +100 mV given over 300 ms at depolarizing of 0.1 Hz to produce the current of NCX. Nickel-sensitive currents with a 10-mM nickel Cl were used to quantify NCX current amplitudes. The external solution contained 5 mM HEPES, MgCl$_2$, 10 mM glucose, and 140 mM NaCl. Additionally, the external solution had dihydropyridine antagonist, 10 µM nitrendipine, 10 µM strophanthidin to inhibit Na$^+$/K$^+$ pump, and 100 µM niflumic acid to impede Ca$^{2+}$-activated Cl currents, and pH was adjusted to 7.4.

Using a step/ramp procedure (where −100 mV was stepped to +20 mV at ambient temperature for 100 ms and then ramped back to −100 mV over 100 ms) $I_{\text{Na-Late}}$ was documented with an external solution comprising 130 mM NaCl, 5 mM CsCl, 1 mM CaCl$_2$, 10 mM HEPES, 1 mM MgCl$_2$, and 10 mM glucose. $I_{\text{Na-Late}}$ current was determined when the current was produced when the voltage was ramped back to −100 mV and was sensitive to 30 µM tetrodotoxin as described previously [23].

An internal solution comprising 130 mM K-aspartate, 20 mM KCl, 1 mM MgCl$_2$(6 H$_2$O), 0.005 mM EGTA, and 10 mM HEPES/KOH (pH 7.3) was used to evaluate the Na$^+$/H$^+$ exchanger current in a whole-cell
model. The external solution contained 3.6 mM CaCl$_2$, 150 mM NaCl, 1.2 mM MgCl$_2$(6 H$_2$O), 5.4 mM KCl, 20 mM glucose, and 5 mM HEPES/NaOH (pH 7.4). At 3 Hz, rat ventricular myocytes were isolated, and 40 ms depolarizing pulses from −45 to 0 mV were used [24].

Intracellular Na\(^+\) and reactive oxygen species (ROS) measurements

The ROS production in the mitochondria and cytosol of the isolated rat ventricular myocytes was evaluated from the three groups using MitoSOX Red (Life Technologies, Grand Island, NY, USA) and CellROX green (Life Technologies), respectively. Moreover, cytosolic Na\(^+\) concentration in the ventricular myocytes was measured using Asante NaTRIUM Green-2 AM (Teflabs). Like our previous investigation[25], an experiment was performed using the Axiovert 100 inverted microscope 63×/1.25 numerical aperture oil immersion objective and the Zeiss LSM 510 with laser scanning confocal microscope (Carl Zeiss). The myocytes were placed in a standard Tyrode's solution containing 5.4 mM KCl, 137 mM NaCl, 0.5 mM MgCl$_2$, 10 mM HEPES, and 1.8 mM CaCl$_2$ with an appropriate fluorescent dye (2 µM MitoSOX Red, 10 µM CellROX green or 5 µM Asante NaTRIUM Green-2 AM). At 488 nm, MitoSOX Red, CellROX green, and Asante NaTRIUM Green-2 AM were stimulated, and their fluorescence signals were captured using the XY mode confocal system at wavelengths >505 nm. Moreover, the myocytes were timed at 1 Hz during the experiment. Using Sigma Plot (version 12) and Image-Pro Plus (version 6.0), fluorescence images were examined as described previously [23].

Western blot

As described, equal quantities of proteins were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and laid down onto the polyvinylidene difluoride membranes [26]. The obtained blots were investigated with antibodies against ryanodine receptor (RyR2;1:5000; Affinity Bioreagents, Golden, CO, USA), phosphorylated RyR2 (pRyR2 (S2808); 1:3000; Badrilla, Leeds, UK), Ca\(^{2+}\) channel, voltage-dependent L-type alpha 1C subunit (Cav1.2; 1:1000; Alomone Labs, Jerusalem, Israel), NCX (1:3000; Swant, Bellizona, Switzerland), Ca\(^{2+}\) uptake via SR Ca\(^{2+}\)-ATPase (SERCA2a; 1:1000; Santa Cruz Biotechnology, CA, USA), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII-δ; 1:2000; Gene Tex, CA, USA), phospholamban (PLB; 1:5000, Thermo Fisher Scientific, MA, USA), and phosphorylated PLB (pPLB)-S16; 1:5000, Badrilla, Leeds, UK). The blots were then exposed to horseradish peroxidase-conjugated secondary antibodies (Leinco Technology) [27]. When binding antibodies were found on an autoradiographic film, band intensities were measured using Alpha Innotech by AlphaEaseFC (San Leandro, CA, USA). Cardiac glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich) was used to standardize and validate equal protein loaded from the targeted bands.

Statistical analysis

The means and standard errors of the means (SEMs) for all quantitative data are displayed. A one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test was conducted (GraphPad Prism version 8.0, San Diego, CA, USA) to identify the significant between-group differences. Statistical significance was defined as a p-value of <0.05.
Results

Electrophysiology of the ventricular myocytes of control rats, DM rats on ND, and DM rats on KD

DM rats on ND had longer QT intervals (80 ± 4 ms) and corrected QT (QTc) intervals (180 ± 9 ms) than either control rats (58 ± 2 and 148 ± 4 ms, respectively; Tukey’s HSD test: both p < 0.005) or DM rats on KD (67 ± 2; Tukey’s HSD test: p < 0.01 and 153 ± 4 ms; Tukey’s HSD test: p < 0.05, respectively; Fig. 2A). The RR intervals were longer in DM rats on ND than in control rats (203 ± 20 vs. 154 ± 2 ms; Tukey’s HSD test: p < 0.05), but the RR intervals between DM rats on ND and KD were similar.

In the ventricular myocytes of DM rats on ND, APD
\[20\], APD
\[50\], and APD
\[90\] were 29.6 ± 2.4, 75.7 ± 5.1, and 173.7 ± 8.1 ms, respectively (n = 18); they were longer than those in the ventricular myocytes of control rats (10.8 ± 1.8, 34.7 ± 4.5, and 135.2 ± 8.2 ms, respectively; n = 13; Tukey’s HSD test: p < 0.005) and of DM rats on KD (21.5 ± 2.1, 59.9 ± 4.1, and 146.5±5.7 ms, respectively; n = 16; Tukey’s HSD test: p < 0.05; Fig. 2B). Compared with control rats, DM rats on KD had ventricular myocytes with longer APD
\[20\] and APD
\[50\] (Tukey’s HSD test: p < 0.005) but with similar APD
\[90\]. Resting membrane potential (RMP) and action potential amplitude (APA) were similar in the control, DM rats on ND, and DM rats on KD groups.

\(\text{Ca}^{2+}\) stores in the ventricular myocytes of DM with or without KD

Next, we assessed \(\text{Ca}^{2+}\) homeostasis in the ventricular myocytes of control rats, DM rats on ND, and DM rats on KD: \([\text{Ca}^{2+}]_{\text{i}}\) transients were 55% and 45% fewer in DM rats on ND (1.0 ± 0.1 F/F, n = 25) than in control rats (1.6 ± 0.2 F/F, n = 37) and DM rats on KD (1.5 ± 0.1 F/F, n = 29), respectively (Tukey’s HSD test: both p < 0.005). However, the control and DM rats on KD had the same \([\text{Ca}^{2+}]_{\text{i}}\) transients (Fig. 3). Additionally, the decay time of \([\text{Ca}^{2+}]_{\text{i}}\) transients in the ventricular myocytes of DM rats on ND was significantly longer (249.6 ± 42.1 ms, n = 20) than that in the ventricular myocytes of control rats (97.5 ± 8.6 ms, n = 31) and DM rats on KD (103.2 ± 12.0 ms, n = 17; Tukey’s HSD test: both p < 0.005). Nevertheless, the control and DM rats on KD ventricular myocytes had similar decay times of \([\text{Ca}^{2+}]_{\text{i}}\) transients (Fig. 3).

As illustrated in Fig. 3, \(\text{Ca}^{2+}\) leak in the ventricular myocytes of DM rats on ND was significantly prolonged (1 ± 0.3 F/F, n = 12) compared with that in the ventricular myocytes of control rats (0.3 ± 0.1 F/F, n = 11) and of DM rats on KD (0.4 ± 0.03 F/F, n = 19; Tukey’s HSD test: both p < 0.05). By contrast, the control and DM rats on KD ventricular myocytes had similar \([\text{Ca}^{2+}]_{\text{i}}\) leak frequency (Fig. 3).

Effects of KD \(I_{\text{Ca-L}}\) current and NCX current in the ventricular myocytes of DM rats with and without KD

\(I_{\text{Ca-L}}\) density (in the peak \(I_{\text{Ca-L}}\) elicited from −50 to 10 mV) in the ventricular myocytes of DM rats on ND was higher (3.7 ± 0.3 pA/pF, n = 17) than that in the control rats (6.6 ± 0.7 pA/pF, n = 16; Tukey’s HSD test: p < 0.005) and DM rats on KD (5.0 ± 0.5 pA/pF, n = 13; Tukey’s HSD test: p < 0.05; Fig. 4A). Figure 4B illustrates the tracings and current-voltage relationships of nickel-sensitive NCX currents in the ventricular
myocytes of control rats, DM rats on ND, and DM rats on KD. We noted a smaller reverse mode of nickel-sensitive NCX currents in the ventricular myocytes DM (0.28 ± 0.03 pA/pF, n = 21) than in the control rats (0.55 ± 0.06 pA/pF, n = 12) and of DM rats on KD (0.49 ± 0.04 pA/pF, n = 12; Tukey’s HSD test: both p < 0.005).

**Effects of KD on I_{Na-late} and Na^+ /H^+ exchanger current on the ventricular myocytes DM rats with or without KD**

I_{Na-late} density of DM rats on ND ventricular myocytes was higher (0.93 ± 0.16 pA/pF, n = 17) than that in the ventricular myocytes of control rats (0.49±0.05 pA/pF, n = 16; Tukey’s HSD test: p < 0.01) and of DM rats on KD (0.56±0.07 pA/pF, n = 13; Tukey’s HSD test: p < 0.05; Fig. 5). Na^+/H^+ exchanger current density of DM rats on ND ventricular myocytes was greater (0.93 ± 0.16 pA/pF, n = 16) than that in the ventricular myocytes of control rats (0.49 ± 0.05 pA/pF, n = 17) and of DM rats on KD (0.56 ± 0.07 pA/pF, n = 13; Tukey’s HSD test: both p < 0.05; Fig. 5).

**Oxidative stress of the ventricular myocytes of the control rats and DM with or without KD**

As shown in Fig. 6, cytosolic ROS levels were onefold higher in the ventricular myocytes of DM rats on ND (102.6 ± 3.4pF/F_0, n = 54) than in the control rats (72.0 ± 7.0pF/F_0, n = 32; Tukey’s HSD test: p < 0.005) and DM rats on KD (85.8 ± 7.1pF/F_0, n = 24; Tukey’s HSD test: p < 0.05). The cytosolic ROS levels of the control and DM rats on KD ventricular myocytes were similar. Moreover, the mitochondrial ROS levels of the ventricular myocytes were 36.6% and 36.2% higher in DM rats on ND (37.3 ± 3.5pF/F_0, n = 24) than in control rats (25.5 ± 11.0pF/F_0, n = 39) and DM rats on KD (28.2 ± 1.3pF/F_0, n = 52; Tukey’s HSD test: both p < 0.005), respectively (Fig. 6). Similarly, intracellular Na^+ ([Na^+]_i) levels in ventricular myocytes were 1.5-fold higher in DM rats on ND (146.3 ± 4.6pF/F_0, n = 42) than in control rats (112.1 ± 5.6pF/F_0, n = 22) and DM rats on KD (125.4 ± 3.5pF/F_0, n = 42; Tukey’s HSD test: both p < 0.005; Fig. 6). Control rats demonstrated 1.2-fold higher [Na^+]_i levels in ventricular myocytes than DM rats on KD.

The effects of Ca^{2+} regulatory proteins in DM hearts with or without KD

Ca^{2+} regulatory protein expression in the ventricles of control, DM rats on ND, and DM rats on KD were analyzed through Western blotting (Fig. 7). Compared with DM rats on ND, those on KD has higher SERCA2a, NCX, and Cav1.2 expression, as well as phosphorylated ryanodine receptor 2 (RyR2) at serine 2808/total RyR2 ratios and phosphorylated phospholamban/total phospholamban ratios (Fig. 7). Moreover, CaMKII-δ expression was higher in DM rats on ND than in control and DM rats on KD (Fig. 7).

**Discussion**

KD may have cardiovascular benefits in DM hearts [4, 28, 29]; however, the results have been controversial [30, 31]. In DM hearts, changes in the excitation-contraction coupling have been noted, which include defects in [Ca^{2+}]_i signaling[32], SERCA2a [33], RyR [34], and NCX [35]. However, whether KD
can ameliorate the electromechanical dysfunction in diabetic cardiomyopathy remains unknown. We discovered in this study that KD administration reversed the electromechanical dysfunction of the hearts of DM rats. More specifically, KD attenuated DM’s effect on ventricular APs, \([\text{Ca}^{2+}]_i\) transients, and \(I_{\text{Na-late}}\) which may be electromechanical mechanisms underlying the antidiabetic cardiomyopathy effects of KD.

DM patients generally demonstrate a high incidence of prolonged QT and QTc interval because of an increment in the APD, making them liable to have an increased incidence of cardiac arrhythmia [36, 37]. Similar to our earlier studies [11, 38], the current DM rats showed a higher heart-to-body weight ratio, prolonged ventricular APD, and a longer QT and QTc interval. The prolonged APD and QT interval aggravate the reduction in the stroke volume and diastolic filling at a higher heart rate [39]. Ventricular arrhythmia may increase because the early afterdepolarization activity will be triggered [38]. In the present study, KD attenuated the prolonged APD and QTc intervals in DM hearts, suggesting that KD has a cardioprotective potential against diabetic cardiomyopathy.

\(\text{Ca}^{2+}\) is responsible for the connection between mechanical contraction and electrical activation and is crucial in excitation-contraction coupling. \(\text{Ca}^{2+}\) from its SR stores through RyR triggers the release of ionic \(\text{Ca}^{2+}\). The ionic \(\text{Ca}^{2+}\) further facilitates \(\text{Ca}^{2+}\) binding to the myofilaments, enhances \([\text{Ca}^{2+}]_i\) content, and activates myocardium contractions [40]. Similar to our previous studies [11, 38], we noted depletion of SR \(\text{Ca}^{2+}\) contents associated with prolonged \([\text{Ca}^{2+}]_i\) decay and relatively few \([\text{Ca}^{2+}]_i\) transients in DM hearts. According to a previous study, reduction in SR \(\text{Ca}^{2+}\) uptake is one of the primary mechanisms causing a diminution in myocardial contractility of diabetic cardiomyopathy [41]. In our DM rats’ ventricular myocytes, the dysfunction in SERCa2a may be ascribed to a reduced protein expression of SERCA2a, leading to transient decay rates and decreased \([\text{Ca}^{2+}]_i\) transient amplitudes [33, 42]. In our DM cardiomyocytes, reduction in \([\text{Ca}^{2+}]_i\) transients may have originated from the reduced SR \(\text{Ca}^{2+}\) content, causing cardiac dysfunction and impairing excitation-contraction coupling efficiency. The impaired reuptake of \(\text{Ca}^{2+}\) during the diastolic phase may have caused SERCA function impairment, causing a decline in SR \(\text{Ca}^{2+}\) stores, which might have worsened the condition of our DM rat hearts due to lowered SERCA2a levels. The decrease in SERCA2a levels might have reduced SR \(\text{Ca}^{2+}\) stores in our DM rat hearts. The prolonged \([\text{Ca}^{2+}]_i\) transient decay in our DM rat hearts might also have been due to SR \(\text{Ca}^{2+}\) depletion. The rise in the transient decay of \([\text{Ca}^{2+}]_i\) might be attributed to the dysfunction of the SERCA pump generating a defect in the cardiomyocytes’ relaxation and a slower removal rate of cytoplasmic \(\text{Ca}^{2+}\) [35, 43].

An increase in \([\text{Ca}^{2+}]_i\) transient decay may also result from prolonging APD in DM ventricular myocytes. We also discovered that KD decreased the \([\text{Ca}^{2+}]_i\) transients and delayed \([\text{Ca}^{2+}]_i\) decay, which diminished \(\text{Ca}^{2+}\) storage in DM hearts. An augmented SERCA2a function in the myocardium could have caused these effects. Furthermore, as measured through echocardiography, the impact of \(\text{Ca}^{2+}\) modulation of KD in DM hearts may have significantly contributed to improving myocardial function. In the current study, KD ameliorated the \(\text{Ca}^{2+}\) dysregulation caused by the negative impact of DM, as indicated by a decrease
in CAMKII-δ expression and an increase in pRyR2 and SERCA2a expressions. The findings suggest that KD improves Ca\(^{2+}\) homeostasis, ameliorating electrical cardiac dysfunction in DM hearts.

\(I_{\text{Ca-L}}\) plays a vital role in inducting the contractile cycle of cardiomyocytes. Inhibition of \(I_{\text{Ca-L}}\) reduces the entry of Ca\(^{2+}\), resulting in a diminution in contractile force and Ca\(^{2+}\) transients [44]. A decreased \(I_{\text{Ca-L}}\) in the ventricular myocytes of our DM rats significantly lowered peak systolic \([\text{Ca}^{2+}]_{i}\), similar to our previous results [42, 45]. Cav1.2 is the principal pathway to enter Ca\(^{2+}\) into the myocardial cells [46]. In our DM cardiomyocytes, Cav1.2 is also altered based on the decreased levels of Cav1.2. Decreased \(I_{\text{Ca-L}}\) density and Cav1.2 expression were restored to control levels in DM rats after KD administration, suggesting that KD may have a cardioprotective role against diabetic cardiomyopathy.

Comparable with the outcomes from other investigations [47–49], our DM rat cardiomyocytes, through voltage-dependent \(I_{\text{Ca-L}}\) demonstrated diminished Ca\(^{2+}\) entry. In our DM rat hearts, the adverse effects on ventricular contractility were caused mainly by the decreased availability of Ca\(^{2+}\) through the \(I_{\text{Ca-L}}\) channel. As indicated in electrocardiograms, this may also have led to QT and QTc interval prolongation. However, the decreased \(I_{\text{Ca-L}}\) in our DM rat hearts was ameliorated in DM rats on KD, contributing to the increase in SR Ca\(^{2+}\) contents and \([\text{Ca}^{2+}]_{i}\) transients and suggesting the cardioprotective effect of KD in diabetic cardiomyopathy. Similarly, we found that NCX function is reduced in DM cardiomyocytes. NCX inhibition in the reverse mode Ca\(^{2+}\) influx of NCX may have limited cellular Ca\(^{2+}\) content and contribute to a decrease in \([\text{Ca}^{2+}]_{i}\), leading to electrical dysfunction.

Moreover, in our DM rat ventricular myocytes, KD ameliorated the diminished NCX current and the chronic impact of KD on the Na\(^{+}/\text{H}^{+}\) exchanger current. The elevation in Na\(^{+}/\text{H}^{+}\) exchanger current and \(I_{\text{Na-late}}\) in our DM rats cardiomyocytes may have contributed to the rise of \([\text{Na}^{+}]_{i}\), causing Ca\(^{2+}\) overload and increasing the risks of oxidative stress and arrhythmia. [50] Our results showed KD reduced intracellular Na\(^{+}\) concentrations and turned back \(I_{\text{Na-late}}\) and Na\(^{+}/\text{H}^{+}\) exchange currents. These findings suggest that KD has cardiovascular benefits in DM hearts.

In the heart’s excitation-contraction coupling, RyR2, a macromolecular homotetrameric protein complex, controls the release of Ca\(^{2+}\) from the SR [46]. CaMKII, one of the accessory proteins involved in RyR2 regulation, interacts with RyR2 and stabilizes the channel, reducing spontaneous Ca\(^{2+}\) release and SR Ca\(^{2+}\) leak[51]. Similar to others, we also noted a significant decrement in the phosphorylated RyR2 at serine 2808/RyR2 ratio in our DM rats' ventricular myocytes [52, 53]. KD may have increased \([\text{Ca}^{2+}]_{i}\) transient amplitude, improved myocardial contractility, and decreased RyR2 phosphorylation in our DM hearts. As observed in our DM rats' ventricular myocytes, a decrease in RyR function prolongs the time to peak Ca\(^{2+}\) transients and slows down Ca\(^{2+}\) release[43]. Because of enhanced RyR2 channel activity in DM hearts, the rise in the SR Ca\(^{2+}\) leak facilitates their arrhythmogenic potential [54]. Increased Ca\(^{2+}\) leak in our investigation might have been associated with diminished phosphorylated RyR2 at serine 2808/RyR2 ratio. This might have caused a reduction in SR Ca\(^{2+}\) content, leading to myocardial
dysfunction. Chronic \([Ca^{2+}]_i\) leak from cardiomyocytes causes an increase in endoplasmic reticulum stress and mitochondrial dysfunction. Mitochondrial dysfunction also increases ROS production and provokes redox modifications of RyR2, thereby exacerbating the leakage of \(Ca^{2+}\) [55]. Consequently, \([Ca^{2+}]_i\) leak inhibition is a critical mechanism of action of KD in DM rats. Thus, KD may help reduce excessive \(Ca^{2+}\) leakage in the SR, resulting in a \(Ca^{2+}\) shortage and causing myocardial dysfunction in diabetic cardiomyopathy.

Heart disorders, including cardiac hypertrophy, arrhythmias, and heart failure, are linked to CaMKII overexpression [56]. CaMKII is an essential mediator for excitation-contraction coupling in the heart because it regulates \(Ca^{2+}\) regulatory proteins [57]. CaMKII activation can cause alterations in \([Ca^{2+}]_i\) signaling, causing impairments in transcriptional regulation, cell mechanics, energetics, and excitation-contraction coupling [56, 58]. CaMKII-δ alters the regulation of \(Ca^{2+}\) by phosphorylating phospholamban and RyR2, affecting the SR \(Ca^{2+}\) content to fluctuate and a leak of diastolic \(Ca^{2+}\),[59] leading to diastolic dysfunction and an increase in arrhythmogenesis [60]. CaMKII-δ inhibition, demonstrated by the decrease in CaMKII-δ level after KD administration, reduces diastolic SR \(Ca^{2+}\) leak in DM rats on KD, resulting in a diminution in the released of spontaneous \(Ca^{2+}\) and an improvement in the SR's capacity to store \(Ca^{2+}\). This suggested the protective role of KD against arrhythmogenicity. Moreover, KD reduces both \([Na^+]_i\) level and CaMKII activity, contributing to reductions in cardiac remodeling and myocardial dysfunction related to diabetic cardiomyopathy.

We also investigated ROS generation in the DM hearts and noted that KD reduced ROS production in both the mitochondrial and cytosol. Mitochondrial ROS can cause local endoplasmic reticulum \(Ca^{2+}\) release events, enhancing the frequency of sparks and \(Ca^{2+}\) leaks in the cardiomyocytes. The reduced ROS generation following KD ingestion may have helped to stop the \(Ca^{2+}\) leak in DM cardiomyopathy. Additional assessment of the mechanical functions to comprehend the cardioprotective impact of KD is necessary for DM cardiomyopathy. The decrement of \(Ca^{2+}\) leak, ROS, \(I_{Na-late}\), and \(Na^+/H^+\) exchanger current in the DM cardiomyocytes on KD suggests that KD may have antiarrhythmic potential. Therefore, further investigation to establish the antiarrhythmic effect of KD is necessary.

Hyperglycemia is linked with a high risk of arrhythmia and myocardial dysfunction. Our study demonstrated several potential factors related to the regulation of \(Ca^{2+}\), which are dysregulated in diabetic cardiomyopathy, including altered expressions of RyR2, phospholamban, SERCA2a, NCX, and CaMKII-δ, and levels of \(I_{Ca-L}\) channel activity. KD alleviated these alterations, possibly improving cardiac cell function in DM. This discovery offers mechanistic insights into diabetic cardiomyopathy’s etiology and might result in more effective tailored treatments for DM patients.

Figure 8 details the possible mechanisms underlying the effects of KD in rats with diabetic cardiomyopathy: KD may restore \(Ca^{2+}/Na^+\) regulation by modifying ionic channels in a DM heart,
resulting in the alleviation of ventricular hypertrophy, reversion of QT interval prolongation, and amelioration of cardiac dysfunction in DM cardiomyocytes.

In conclusion, as summarized in Fig. 8, DM-induced Ca\(^{2+}\) or Na\(^{+}\) dysregulation in the cardiomyocytes was restored in the hearts of KD-treated rats by reducing ionic channel modification and ROS, resulting in improved myocardial function, ventricular hypertrophy reduction, and prolonged QT interval correction.

Declarations

Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee Panel of Taipei Medical University reviewed and approved our animal experimentation protocols with the IACUC/IACUP approval number LAC 2020-0478.

Consent for publication

All authors have read and approved the final version of the manuscript.

Availability of data and materials

The article's data and online supplementary material are available in the paper.

Competing interests

On behalf of all authors, the corresponding author confirms that no known conflicts of interest are associated with this publication, and no significant financial support for this work could have influenced its outcome. It states that there are no competing interests.

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Authors' contributions

YJC, YCC, TIL, and NNT contributed to the scheme and conceptualization of this study. TIL, NNT, and TWL performed the experiments. YHK and SH participated in acquiring and interpreting data for the work. TWL and NNT provided the manuscript and figures. TIL and YCC created the graphical abstracts. YJC and TIL reviewed and edited the final manuscript. All authors have read and approved the final manuscript.
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Figures

![Experimental Design](image)

Figure 1

Schematic of our experimental design. Abbreviations: KD: ketogenic diet, DM: diabetes mellitus, ND: normal diet, Ca^{2+}: calcium, SR: sarcoplasmic reticulum, Na^{+}: sodium.
Figure 2

Changes in the electrocardiogram and action potentials (APs) of ventricular myocytes in control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on the ketogenic diet (DM+KD). (A) Average data and representative tracings of the electrocardiograms before and after treatment in ventricular myocytes of control (N = 7), DM+ND (N = 7), and DM+KD (N = 7). (B) Average data and representative AP tracings in the ventricular myocytes of control (n = 13), DM+ND (n = 18), and
DM+KD (n = 16). Abbreviations: QT: QT interval, RR: RR interval, QTc: corrected QT interval, N: number of rats, n: number of cardiomyocytes isolated from the rats, RMP: resting membrane potential, APA: action potential amplitude, APD$_{20}$, APD$_{50}$, and APD$_{90}$: action potential durations of repolarization at 20%, 50%, and 90% respectively. Statistical significance was assessed using a one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test. * p < 0.05; *** p < 0.005.

**Figure 3**

[Ca$^{2+}$]$_i$ transients, Ca$^{2+}$ stores measured from caffeine (20 mM)-induced Ca$^{2+}$ transients, and Ca$^{2+}$ leak in the ventricular myocytes of control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on the ketogenic diet (DM+KD). (A) Average data and representative tracings of [Ca$^{2+}$]$_i$ transients and decay of time of [Ca$^{2+}$]$_i$ transients in the ventricular myocytes of control (n = 37), DM+ND (n = 25), and DM+KD (n = 29). (B) Average data and representative tracings of Ca$^{2+}$ stores in the ventricular myocytes of control (n = 31), DM+ND (n = 20), and DM+KD (n = 17). (C) Average data and representative tracing of the incidence and frequency of Ca$^{2+}$ leak in the ventricular myocytes of control (n = 11), DM+ND (n = 12), and DM+KD (n = 19). Abbreviations: [Ca$^{2+}$]$_i$: intracellular calcium, Ca$^{2+}$:calcium, n: number of cardiomyocytes isolated from the rats. Statistical significance was assessed using a one-
way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test. * \( p < 0.05 \); *** \( p < 0.005 \).

**Figure 4**

\( I_{\text{Ca-L}} \) and NCX current in ventricular myocytes of control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on the ketogenic diet (DM+KD). (A) Representative tracings of \( I_{\text{Ca-L}} \) current and current-voltage relationship of \( I_{\text{Ca-L}} \) in the ventricular myocytes of control \( (n = 16) \), DM+ND \( (n = 14) \), and DM+KD \( (n = 13) \). (B) Representative tracings of NCX current and current-voltage relationship of NCX current in the ventricular myocytes of control \( (n = 12) \), DM+ND \( (n = 21) \), and DM+KD \( (n = 12) \) rats.

Abbreviations: \( I_{\text{Ca-L}} \): L-type calcium current, NCX: sodium/calcium exchanger, n: number of cardiomyocytes isolated from the rats. Statistical significance was assessed using a one-way analysis of variance (ANOVA) with Tukey’s Honest Significant Difference (HSD) test. \# \( p < 0.05 \) vs the controls; #### \( p < 0.01 \) vs the controls; *** \( p < 0.005 \) vs DM+KD rats.
Figure 5

$I_{Na-Late}$ and Na$^+/H^+$ exchanger current in ventricular myocytes of control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on the ketogenic diet (DM+KD). (A) Average data and representative tracing of $I_{Na-Late}$ current in the ventricular myocytes of control ($n = 17$), DM+ND ($n = 16$), and DM+KD ($n = 13$). (B) Average data and representative tracings of Na$^+/H^+$ exchanger current in the...
ventricular myocytes of control (n = 11), DM+ND (n = 14), and DM+KD (n = 14). Abbreviations: $I_{\text{Na-Late}}$: late sodium current, $\text{Na}^+/\text{H}^+$ exchanger: sodium/hydrogen exchanger, n: number of cardiomyocytes isolated from the rats. Statistical significance was assessed using a one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test. ** $p < 0.01$ vs control; * $p < 0.05$ vs DM+KD.

Figure 6

Oxidative stress and cytosolic sodium ($\text{Na}^+$) levels in the ventricular myocytes of control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on the ketogenic diet (DM+KD). (A) Average data of cytosolic ROS levels and examples of cytosolic ROS in the ventricular myocytes of control (n = 31), DM+ND (n = 54), and DM+KD (n = 24). Scale bar, 10mm. (B) Average data of mitochondrial ROS levels and examples of mitochondrial ROS in the ventricular myocytes of control (n = 39), DM+ND (n = 24), and DM+KD (n = 52). Scale bar, 10mm. (C) Average data of cytosolic $\text{Na}^+$ levels and an example in the ventricular myocytes of control (n = 22), DM+ND (n = 42), and DM+KD (n = 42). Scale bar, 10mm Abbreviations: ROS: reactive oxygen species, n: number of cardiomyocytes isolated from the
rats. Statistical significance was assessed using a one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test. * \(p < 0.05\); *** \(p < 0.005\).

**Figure 7**

Calcium regulating proteins in the ventricular myocytes of control rats (control), diabetes mellitus (DM) rats on a normal diet (DM+ND), and DM rats on a ketogenic diet (DM+KD). Average data and representative immunoblots of (A) RyR2 phosphorylated at serine 2808/RyR2 ratio (N = 4 per group), (B) NCX (N = 5 per group), (C) SERCA2a (N = 5 per group), (D) Cav1.2 (N = 6 per group), (E) CaMKII-d (N = 6 per group), and (F) phosphorylated PLB/PLB ratio (N = 4 per group) in ventricular myocytes of control,
DM+ND, and DM+KD. Densitometry was normalized to GAPDH as the internal control. Abbreviations: RyR2: ryanodine receptor 2, NCX: sodium/calcium exchanger, SERCA2a: sarcoplasmic reticulum calcium ATPase, Cav1.2: voltage-dependent L-type alpha 1C subunit, CaMKII-d: calcium/calmodulin-dependent protein kinase II, PLB: phospholamban. N: number of rats. Statistical significance was tested using a one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test. * p < 0.05.

Figure 8

Schematic diagram of the proposed mechanism of action underlying the effects of KD in DM hearts. KD may reverse DM-induced Ca$^{2+}$/Na$^{+}$ dysregulation by reducing ROS and ionic channel modification in the cardiomyocytes, leading to improved cardiac function and correction of prolonged QTc intervals. Abbreviations: APD: action potential duration, DM: diabetes mellitus, Ca$^{2+}$: calcium, Cav1.2: Ca$^{2+}$channel, voltage-dependent, L-type alpha 1C subunit, H$: hydrogen, [Na$^{+}$]: intracellular sodium, I$_{Na\text{-Late}}$: late sodium current, Na$: sodium, NCX: Na$^{+}$/Ca$^{2+}$ exchanger, QTc: corrected QT interval, ROS: reactive oxygen species, RyR2: ryanodine receptor 2, pRyR2: phosphorylated RyR2, SR: sarcoplasmic reticulum, SERCA: sarcoplasmic reticulum ATPase, STZ: streptozotocin.
Supplementary Files

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- SupplementaryData202305.docx