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Thinned young apple polyphenol prevents diabetic cardiomyopathy through promoting TET2-mediated active DNA demethylation in STZ-induced diabetic mice heart

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ABSTRACT

Diabetic cardiomyopathy (DCM) refers diabetic patients develop cardiomyopathy characterized by cardiac systolic and diastolic dysfunction. Currently, no research has explored the role of ten-eleven translocation (TET)-mediated active DNA demethylation in the development of DCM. Through case-control study, we found that the level of 5-hydroxymethylcystein in peripheral blood DNA of DCM patients was significantly lower than it in the healthy subjects and diabetic patients. Secondly, we had conducted an animal study to explore the effect of thinned young apple polyphenol (TYAP) on DNA demethylation in streptococci-induced diabetic mice. TYAP effectively prevented the ventricular dysfunction and cardiomycyte disarray *via* alleviating DNA epigenetic modifications metabolic disorders in diabetic mice heart. TYAP increased the stability of TET2 protein *via* activating phosphorylate AMPK and enhanced the activity of TETs enzymes *via* improving tricarboxylic acid cycle, then promoted TET-mediated active DNA demethylation. TYAP prevented the occurrence of DCM by promoting TET2-mediated active DNA demethylation in the heart of diabetic mice.

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1. Introduction

Diabetic cardiomyopathy (DCM) refers to patients with diabetes accompanied by cardiac systolic and diastolic dysfunction, which is one of the leading causes of death among various complications of diabetes[1-2]. The prevalence of DCM is 1.1% in community population, but the prevalence of DCM ranging from 19%–26% in patients with diabetes[3-4]. The number of DCM patients is increasing rapidly in parallel with the increase in diabetes. Therefore, it is urgent to find the effective strategies to prevent DCM in diabetic patients, which has important public health significance.

It is now clear that epigenome is needed for the maintenance of heart function under normal conditions and in response to stress[5-6]. In line with this, previous studies found that active DNA demethylation played a vital role in the pathological mechanisms of DCM[7-8]. Active DNA demethylation in mammals is achieved through ten-eleven translocation (TET)-mediated oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), followed by replication-dependent dilution of oxidized 5mC or thymine DNA glycosylase (TDG)-mediated excision of 5fC and 5caC coupled with base excision repair[9-10]. Additionally, recent studies have also reported that promoting TET2 activity in heart may be an effective strategy for improving heart failure[11-12]. It is well

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known that TET2 activity depends on the levels of α-ketoglutaric acid (α-KG) and α-KG inhibitor (succinic acid and fumaric acid), which are the intermediate metabolites of tricarboxylic acid cycle (TCA) and play key role in maintaining mitochondrial function[13]. In addition, adenylate-activated protein kinase (p-AMPK) phosphorylates TET2 at serine residue 97 and 99, and the phosphorylation enhances the protein stability of TET2[14-15]. In this regard, promoting TET2-mediated active DNA demethylation through improving mitochondrial function and AMPK in heart is an effective strategy for preventing DCM.

Thinning young apples (around 1 month after blossom) are usually discarded in orchards to guarantee the output and increase the quality of harvested apples[16]. Noticeably, thinned young apples is considered as a valuable source of apple polyphenol to be used in health care products to prevent or treat heart disease[16-18]. As reported previously, apple polyphenols displayed a robust potential of counteracting inflammation and oxidative stress by improving mitochondrial function and AMPK[19-21]. Additionally, thinned young apple polyphenols (TYAP) effectively improved diabetes-induced neuronal apoptosis *via* up-regulating the expression and activity of TET2[22].

Taken together, the aim of present study was to investigate preventive effect of TYAP on DCM in STZ-induced diabetic mice, and further explored the underlying molecular mechanism from perspective of promoting TET-mediated active DNA demethylation. In order to achieve above-mentioned purpose, we firstly examined the association between the levels of DNA epigenetic modifications and heart function using a case-control study. Secondly, we assessed the prevent effects of TYAP supplementation on heart function by echocardiographic examination in STZ-induced diabetic mice, and further explored molecular mechanism from the perspective of promoting TET2-mediated active DNA demethylation.

2. Material and methods

2.1 Participants and clinical laboratory tests

The study was approved by the Ethics Committee of Qingdao University School of Medicine (QDU-HEC-2022282). In total, 105 participants, including DCM patients, diabetes patients with normal cardiac function, and age-matched health control people, were recruited from 2021 to 2022 in the Health-center of Licha Town, Jiaozhou City, Qingdao, China. Diabetes was defined as fasting blood glucose (FBG) ≥ 7.0 mmol/L or 2 h oral glucose tolerance test (OGTT) ≥ 11.1 mmol/L. DCM was defined as diabetes complicated with myocardial injury, and exclude myocardial injury due to other causes. According to the US guidelines for the fourth general definition of myocardial infarction and previous literature, myocardial injury is considered when the patient has ≥ 2 leads of ST-segment elevation and/or T-wave flat, inverted, or biphasic on the same electrocardiograph[23-24]. When myocardial injury occurs, the key myocardial enzymes including creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) will penetrate into the blood. Therefore, the activity of CK, LDH and AST are used as the marker of myocardial injury. All the diabetes and DCM participants were included in chronic diabetes management in the Health-center of Licha Town. The serum concentrations of total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), CK, ALT, AST and blood glucose were detected by the automatic analyzer.

Sample size calculation was done using the case-control sample size calculation formula:

$$n = \frac{[Z_\alpha \sqrt{2\bar{p}(1-\bar{p})} + Z_\beta \sqrt{p_1(1-p_1)+(1-p_0)}\,]^2}{(p_0-p_1)} \quad (1)$$

$$\bar{p} = \frac{p_0+p_1}{2} \quad (2)$$

Where, n represents the required sample size for each group, specifically the number of subjects needed in either the case group or the control group, assuming equal sample sizes between the two groups; Z_α denotes the critical value corresponding to the type I error rate α under the standard normal distribution; Z_β refers to the critical value associated with the type II error rate β under the standard normal distribution.

In previous studies, the prevalence of heart failure among diabetic patients was 26%[24], which means $p_1 = 0.26$. The prevalence of heart failure among adult was 0.51%[25], which means $p_0 = 0.005\ 1$. Accordingly, the minimal proper sample size was 35 participants in each group to taking into account a α risk at 5% and a β risk at 10%. Therefore, included 35 controls, 35 diabetes patients and 35 DCM patients meet the need of sample size in the present study.

2.2 Animals and experiment design

Overall, 80 of C57BL/6J male mice (8 weeks, 20−25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; license number: SCXK (Jing) 2016-0006). After 1 week of adaptive feeding, the mice were randomly divided into 2 groups as followed: normal control group (CON, n = 15), were injected with 0.5% carboxymethylcellulose sodium lubricant (CMC-Na) solution for 1 week; and streptozotocin group (STZ, n = 65), were injected with streptozotocin at 55 mg/(kg·day) for 1 week. The mice with FBG levels > 11.1 mmol/L were selected as diabetic mice and 60 diabetic mice were obtained (60 of 65, 92.3%). The diabetic mice were randomly divided into the following 4 groups: 1) DCM group (DCM, n = 15), the mice were given 0.5% CMC-Na solution by intragastric administration every day; 2) metformin group (MET, n = 15), the mice were given 150 mg/(kg·day) metformin by intragastric administration; 3) low-dose TYAP group (LAP group, n = 15), the mice were given 150 mg/(kg·day) TYAP by intragastric administration; 4) high-dose TYAP group (HAP group, n = 15), the mice were given 300 mg/(kg·day) TYAP by intragastric administration. During the intervention, diabetic mice were fed high-fat diet (60% kcal fat content, D12492), and the mice in CON group were fed standard diet (10% kcal fat content, D12450J) and 0.5% CMC-Na solution by intragastric administration every day.

All animals were housed in a temperature (22−28 °C) and humidity (50%−60%) controlled room and maintained on a 12 h light/dark cycle (08:00−20:00 light, 20:00−08:00 dark) with enough food and water during the experiments, and their body weight and food intake were observed weekly. After the intervention, the mice were fasted overnight and sacrificed. The hearts and serum of mice were collected. Hearts were rapidly frozen in −80 °C liquid nitrogen or directly stored in 4% paraformaldehyde for histological analysis. All efforts were made to minimize animal suffering. The animal experiment procedures adhered to Qingdao University Guide for the care and use

of Laboratory Animals and approved by Ethics Committee Medical College of Qingdao University (QDU-AEC-2022462).

2.3 Intraperitoneal glucose tolerance test

The intraperitoneal glucose tolerance test (IPGTT) experiment was consistent with the previous experiments of the research group[26]. Mice fasted for 8 h after intraperitoneal injection of glucose. After injection, fasting blood glucose and blood glucose (0, 30, 60, 90 and 120 min) were measured using a commercial blood glucose meter (Roche Active). The area under the IPGTT curve were measured by software (GraphPad Prism 8, USA).

2.4 Cardiac ultrasound examination

After anesthesia with isoflurane in mice, transthoracic echocardiography was performed using Vevo2100 imaging system (VisualSonics, Toronto, Canada)[27]. Left ventricular ejection fraction (EF), fractional shortening (FS) and left ventricular interventricular septum thickness (IVS) and left ventricle volume (LV vol) were measured by M-mode echocardiography in the left ventricular long-axis view:

$$\mathrm{EF}(\%) = \frac{\text{Left ventricular end-diastolic volume} - \text{Left ventricular end-systolic volume}}{\text{Left ventricular end-diastolic volume}} \times 100 \quad (3)$$

$$\mathrm{FS}(\%) = \frac{\text{Left ventricular end-diastolic diameter} - \text{Left ventricular end-systolic diameter}}{\text{Left ventricular end-diastolic diameter}} \times 100 \quad (4)$$

The early (E) and late (A) diastolic mitral flow velocities were measured by pulsed Doppler in the 4-chamber view and the ratio of E/A was calculated. The early (E′) and late (A′) diastolic mitral annular velocities were measured by tissue Doppler imaging in the 4-chamber view and the ratio of E′/A′ was derived. The left ventricular mass index was calculated as follows:

$$\text{The left ventricular mass index} = \frac{\text{The left ventricular mass index}}{\text{Tibia length}} \quad (5)$$

2.5 Blood samples and biochemical measurements

At the end of experimental period, mice were fasted overnight and sacrificed. The serum samples were separated from blood by centrifugation for further analysis. The activities of CK, LDH and AST were measured by an automatic biochemical analyzer (Hitachi 3100) following the commercial kit manufacturer's protocol, and the AST, CK and LDH standards were purchased from MedicalSystem (Ningbo, China). The level of insulin in serum was measured by commercial ELISA kit following the manufacturer's protocol (Jingkang, Shanghai, China). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows:

$$\text{HOMA-IR} = \frac{\text{Fasting insulin concentration} \times \text{Fasting glucose concentration} \times 0.055\,51}{22.5} \quad (6)$$

The levels of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in heart were examined by ELISA kit following the following the manufacturer's protocol (Huijia Biology company, Guangzhou, China).

2.6 Histological analysis of mice heart, genomic DNA extraction and dot-blot

Select the same area for each slide and count the number of myocardial cells in hematoxylin-eosin (H&E) staining. The basic principle of detecting myocardial fibrosis is that long-term chronic myocardial injury caused fibrosis of the endocardium and subendocardial myocardium. In Masson staining, normal myocardium appeared red color, while fibrotic myocardial cells appeared blue. Then ImageJ software was used to calculate the area of red and blue and obtain the percentage, which was the percentage of fibrosis[28-29]. Three non-overlapping fields from each section of heart were randomly chosen and photographed using an Olympus BX53F (Tokyo, Japan) optical microscope. Genomic DNA of heart was extracted according to the TIANamp Genomic DNA kit instructions. The concentration of DNA was determined by NanoDrop (Thermo Scientific, Rockford, IL, USA). Dot-blot was performed as our previously described[30]. The ImageJ and Image-Pro Plus 6.0 software were used to analyze and quantify histopathological and dot-blot results.

2.7 Western blot analysis

Western blot was performed as previous described[31]. In present study, antibodies against AMPKα (Cat#5831, RRID:AB_10622186), p-AMPKα (Cat#2535, RRID: AB_331250), acetyl-CoA carboxylase (ACC) (Cat# 3676, RRID: AB_2219397), p-ACC (Cat# 11818, RRID: AB_2687505), TET2 (Cat# 36449, RRID: AB_2799102), isocitrate dehydrogenase (IDH1) (Cat# 8137, RRID: AB_10950504), IDH2 (Cat# 56439, RRID: AB_2799511), α-ketoglutarate dehydrogenase complex (OGDH) (Cat# 26865, RRID: AB_2737585), succinate dehydrogenase A (SDHA) (Cat# 5839, RRID: AB_10707493), malate dehydrogenase 2 (MDH2) (Cat# 11908, RRID: AB_2797764) and aconitase 2 (ACO2) (Cat# 6922, RRID: AB_10828218) were purchased from Cell Signaling Technology. Antibodies against TET1 (Cat# 61741, RRID: AB_2793752) and TET3 (Cat# 61743, RRID: AB_2793753) were purchased from Active Motif. Antibody for GAPDH was purchased from Boster. Antibodies against citrate synthase (CS) (Cat# A5713, RRID: AB_2766471), DNA methyltransferases 3A (Dnmt3A) (Cat# A3169, RRID: AB_2764959) and Dnmt3B (Cat# A2899, RRID: AB_2764719) were purchased from ABclonal. Antibodies against Dnmt1 (Cat# ET1702-77, RRID: AB_3070337) and fumarate hydratase (FH) (Cat# ER1902-01, RRID: AB_3069386) were purchased from Huabio and the SDHB (Cat#459230, RRID: AB_2532233) was purchased from Invitrogen.

2.8 Metabolite extractions and LC-MS/MS analysis

The heart tissues were cut on ice (approximately 30 mg) into an Eppendorf tube (1.5 mL). To extract metabolites form heart sample, 100 μL of pre-cooled extraction buffer (acetonitrile:methanol: water = 2:2:1, *V/V*) was added, and adequately vortex by a tissue homogenizer (60 Hz, 60 s, twice). Equal volume of acetonitrile containing 0.2% acetic acid (pre-cooled at −20 °C) was added to sample, and adequately vortexed then centrifuged at 17 000 × *g* for 10 min at 4 °C. For LC-MS analysis, the supernatant was filtered with 0.45 μm filter (organic phase).

The concentration of TCA cycle intermediate metabolites were measured by UPLC-MS using an Agilent 1290 Infinity LC system coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent, USA). Chromatographic separations were performed using a ZORBAX SB-C_{18} (2.1 mm × 100 mm, 5 μm, Agilent, USA) and maintained at 35 °C. The mobile phase consisted of water modified with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). The following gradient program was used: 20% B at 0–2 min, 25% B at 4 min; 30% B at 6 min; 90% B at 10–12 min; 20% B at 12.1–15 min. The flow rate was 0.7 mL/min and the injection volume was 5 μL. Mass spectrometry was operating in negative ion mode, the ionization voltage was 4 500 V, the drying gas flow was 10 L/min, and the gas temperature was 200 °C. The nebulizer pressure was set at 40 psi, and the fragment voltage was set at 115 V. Data were collected in a centroid mode and the mass range was set at *m*/*z* 40−1 000 using an extended dynamic range. MultiQuant or Analyst was used for quantitative data processing. The quality control samples (QCs) were processed together with the biological samples. Metabolites in QCs with coefficient of variation less than 30% were denoted as reproducible measurements.

2.9 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The statistical significance of body weight was evaluated with two-way analysis of variance, the correlation between the levels of people blood DNA 5mC and 5hmC and serum myocardial enzyme were analyzed by the Pearson correlation, and other indicators were analyzed by One-way ANOVA (GraphPad Prism 8, USA). The level of significance was set at $P < 0.05$ in all comparisons.

3. Results

3.1 The characteristics of participants

The characteristics of participants were shown in Table 1. There were no obvious difference in age, sex, body mass index (BMI), body fat rate, diastolic blood pressure, heart beat, and the concentration of serum TC, TG, AST, ALT, HDL-C and LDL-C among 3 groups. However, systolic blood pressure level in DCM group was significantly higher than that in CON group and DM group ($P < 0.05$). The FBG level in DM group and DCM group was significantly higher than it in CON group ($P < 0.05$). In addition, the activities of CK and LDH in DCM group were significantly higher than them in CON group and DM group ($P < 0.05$).

3.2 The levels of 5mC and 5hmC in peripheral blood DNA of participants

The levels of 5mC and 5hmC in peripheral blood DNA of participants were detected by dot-blot. As shown in Figs. 1A and C, there was no obvious difference of 5mC levels among CON, DM and DCM groups. However, the level of 5hmC in DCM group was significantly lower than it in CON group ($P < 0.05$) and DM group ($P = 0.062\ 1$) (Figs. 1B and D). By correlated analysis of serum myocardial enzyme profiles with 5mC or 5hmC levels, no significant correlation was found between 5mC level and serum AST ($R^2 = 0.012\ 7$, $P = 0.349\ 2$), CK ($R^2 = 0.000\ 8$, $P = 0.811\ 0$) and LDH ($R^2 = 0.010\ 3$, $P = 0.404\ 9$) activities (Figs. 1E-G). However, there was a significant negative correlation between 5hmC level and serum AST ($R^2 = 0.223\ 3$, $P < 0.001$), CK ($R^2 = 0.530\ 0$, $P < 0.001$), and LDH ($R^2 = 0.545\ 2$, $P < 0.001$) activities (Figs. 1H, I and J).

3.3 TYAP improved diabetes related index in STZ-induced diabetic mice

After 1 week of STZ injection, only the mice with FBG higher than 11.1 mmol/L were diagnosed as diabetic mice in the STZ group (Fig. 2B). During the intervention, the body weight of CON group mice was obvious higher than other groups, but there was no significant difference among DCM, MET, LAP and HAP groups (Fig. 2C). In addition, there was no significant difference in food intake among all groups (Fig. 2D). After intervention, although there was no obvious difference among LAP, HAP and DCM group in the area under

Table 1
Characteristics of participants.

Demographic variable	CON ($n = 35$)	DM ($n = 35$)	DCM ($n = 35$)	*P*
Age (year)	71.91 ± 5.24	70.74 ± 5.40	71.20 ± 4.83	0.206
Male (*n*, percentage (%))	13, 37.14	11, 33.33	15, 42.86	0.402
BMI (kg/m^2)	24.41 ± 2.70	24.59 ± 2.74	25.37 ± 4.44	0.454
Body fat rate (%)	26.99 ± 8.40	28.80 ± 6.90	28.89 ± 8.51	0.549
FBG (mmol/L)	5.27 ± 0.46	9.19 ± 3.08	7.85 ± 3.40	0.000
BP (systolic; mm Hg)	126.43 ± 18.64	136.77 ± 23.86	142.63 ± 28.03	0.019
BP (diastolic; mm Hg)	72.11 ± 10.53	72.11 ± 11.93	77.89 ± 12.78	0.066
Heart beat	66.83 ± 7.48	71.03 ± 9.29	68.97 ± 14.23	0.266
LDH (U/L)	140.49 ± 27.50	157.28 ± 40.78	303.72 ± 173.31	0.004
CK (U/L)	60.90 ± 25.48	65.20 ± 45.77	110.05 ± 82.78	0.001
AST (U/L)	24.20 ± 5.59	23.06 ± 8.21	27.77 ± 14.66	0.137
ALT (U/L)	20.60 ± 8.19	21.69 ± 8.08	23.60 ± 13.30	0.460
TG (mmol/L)	1.16 ± 0.71	1.72 ± 1.01	1.75 ± 1.84	0.098
TC (mmol/L)	4.93 ± 0.97	5.15 ± 1.03	4.94 ± 0.83	0.548
HDL (mmol/L)	1.91 ± 0.27	1.89 ± 0.46	1.93 ± 0.45	0.930
LDL (mmol/L)	3.22 ± 0.93	3.37 ± 0.78	3.14 ± 0.72	0.492

Note: BP, blood pressure.

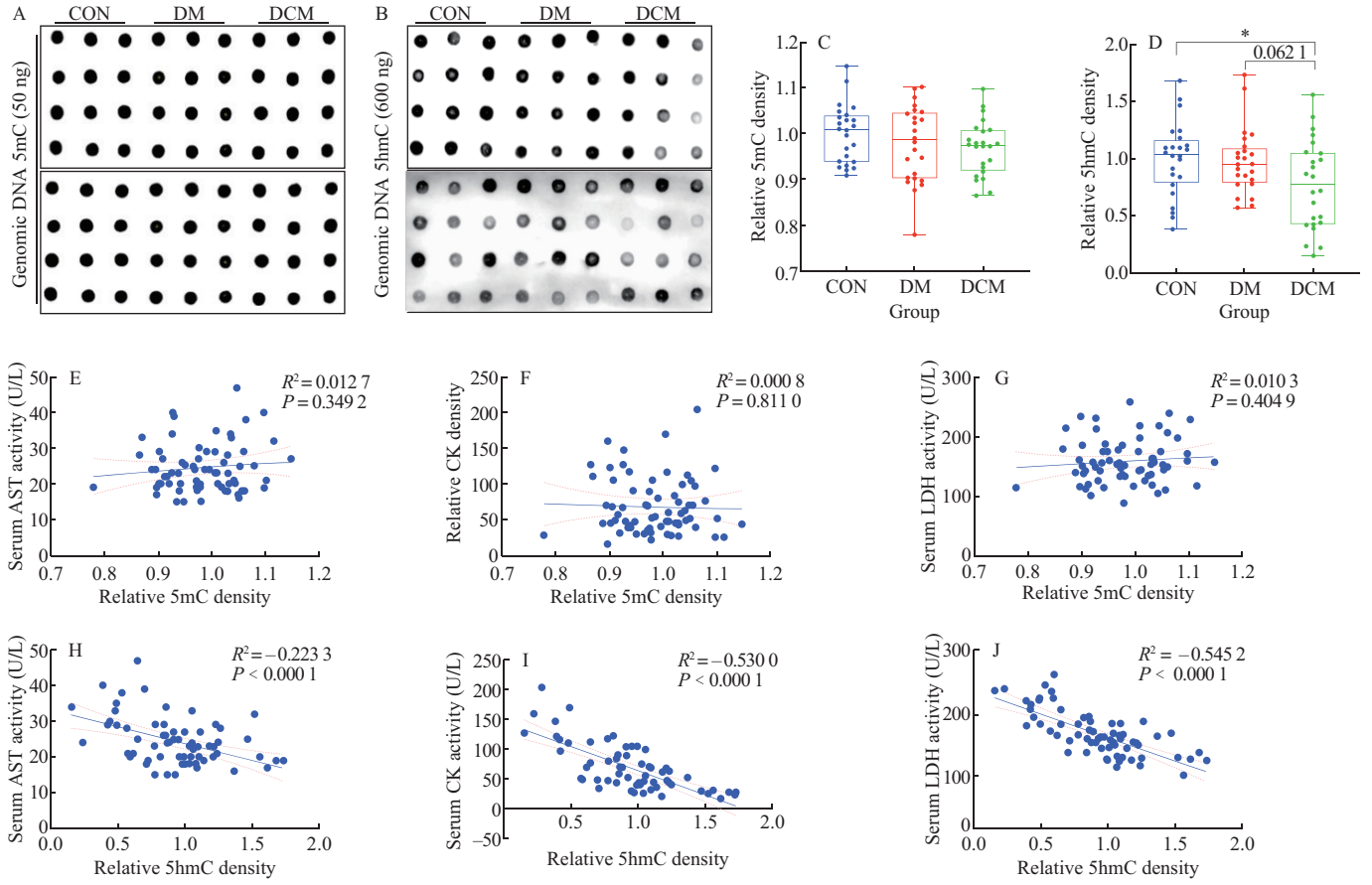

Fig. 1 People blood DNA 5mC and 5hmC levels and the correlation with serum myocardial enzyme profiles. (A) Dot-blotting images of 5mC; (B) dot-blotting images of 5hmC; (C) statistical analysis results of DNA 5mC; (D) statistical analysis results of DNA 5hmC; (E-J) correlation analysis of DNA 5mC and 5hmC were correlated with serum marker. The values are presented as the means ± SEM (n = 24); $^{*}P < 0.05$.

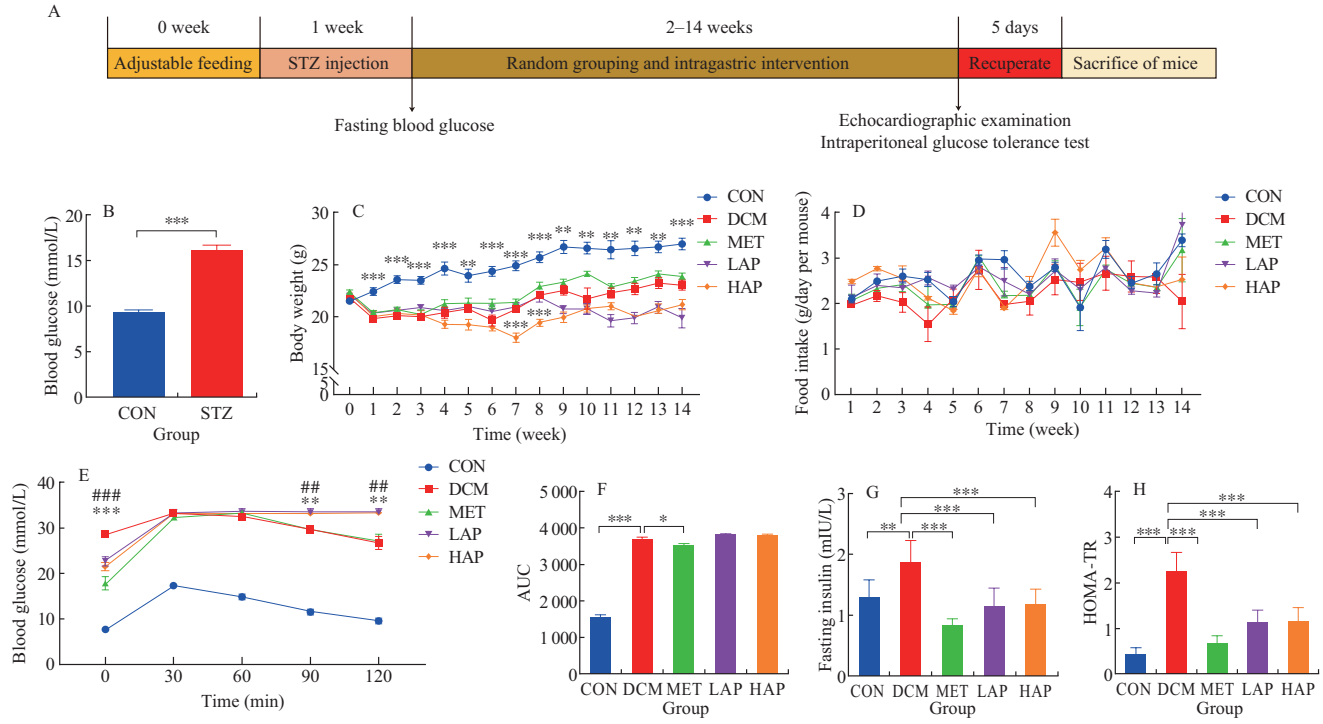

Fig. 2 Effect of TYAP on the parameters of STZ-induced diabetic mice. (A) Schematic diagram of the animal feeding process; (B) blood glucose at the end of modelling; (C) body weight weekly; (D) food intake weekly; (E) blood glucose in the IPGTT; (F) area under the blood glucose curve; (G) fasting insulin; (H) indexes of HOMA-IR. The values were presented as the means ± SEM ($n \geq 6$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ mean other group compared to DCM group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ mean HAP group compared to CON group.

the curve (AUC) of IPGTT (Figs. 2E and F), the levels of fasting insulin and HOMA-IR in the LAP and HAP groups were significantly lower than those in the DCM group ($P < 0.05$) (Figs. 2G and H). These results suggested that TYAP intervention effectively improved insulin sensitivity of diabetic mice.

3.4 TYAP improved cardiac function in STZ-induced diabetic mice

In present study, the cardiac function of mice was detected by cardiac ultrasound. The echocardiogram results showed that EF, FS, E/A and E′/A′ in DCM group were significantly lower than these in CON group ($P < 0.05$) (Figs. 3A-G). These results indicated that diabetic mice were development to DCM during the intervention in present study. However, compared with DCM group, the levels of EF, FS, and E′/A′ and leftventricular mass index were significantly improved in HAP group (Figs. 3D, E and G). Similarly, the levels of EF, E/A, E′/A′ and leftventricular mass index in the LAP group were significantly higher than them in the DCM group ($P < 0.05$) (Figs. 3D, F, G and L). Additionally, compared with DCM group, the levels of diastolic interventricular septum (IVS; d), systolic diastolic interventricular septum (IVS; s), systolic left ventricle volume (LV vol; s) and diastolic left ventricle volume (LV vol; d) were improved by TYAP supplementation although there was no significant effect (Figs. 3H-K).

Except cardiac ultrasound, we also measured the changes of cardiopathology by H&E and Masson staining. As shown in Fig. 4A, the cardiomyocytes of mice in DCM group were arranged in a disordered manner and appeared with obvious gaps than that in CON group, and these characteristics significantly improved in the TYAP supplementation groups. The number of cardiomyocytes was significantly lower in DCM group than in the other groups (Fig. 4C). The results of Masson staining showed that the level of myocardial fibrosis in LAP and HAP group was significantly lower than it in DCM group mice ($P < 0.05$) (Figs. 4B and D). In addition, the activities of CK, LDH and AST in the CON group were significantly lower than them in DCM group ($P < 0.05$) (Figs. 4E-G). Taken together, above these results suggested that TYAP effectively improved diabetic-induced cardiac dysfunction, and then prevented the occurrence of DCM.

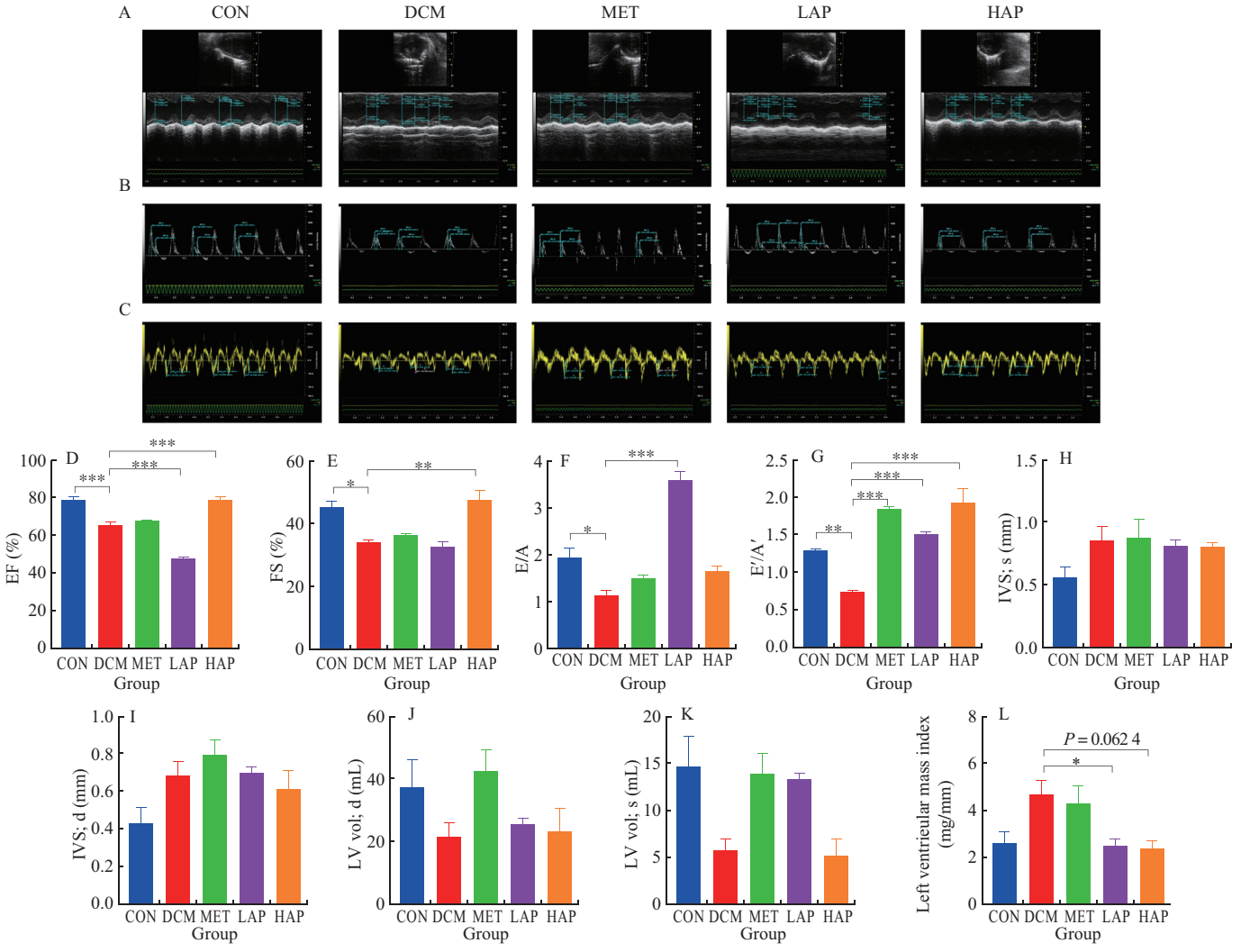

Fig. 3 TYAP prevented the development of DCM in the heart of STZ-induced diabetic mice. (A-C) Echocardiographic testing of mice; (D) EF; (E) FS; (F) mitral peak velocity ratio of early-diastolic filling to atrial contraction (E/A); (G) mitral annular peak velocity ratio of early-diastolic filling to atrial contraction (E′/A′); (H) systolic diastolic IVS; s; (I) diastolic IVS; d; (J) diastolic LV vol; d; (K) systolic LV vol; s; (L) left ventricular mass index. The values are presented as the mean ± SEM ($n \geq 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

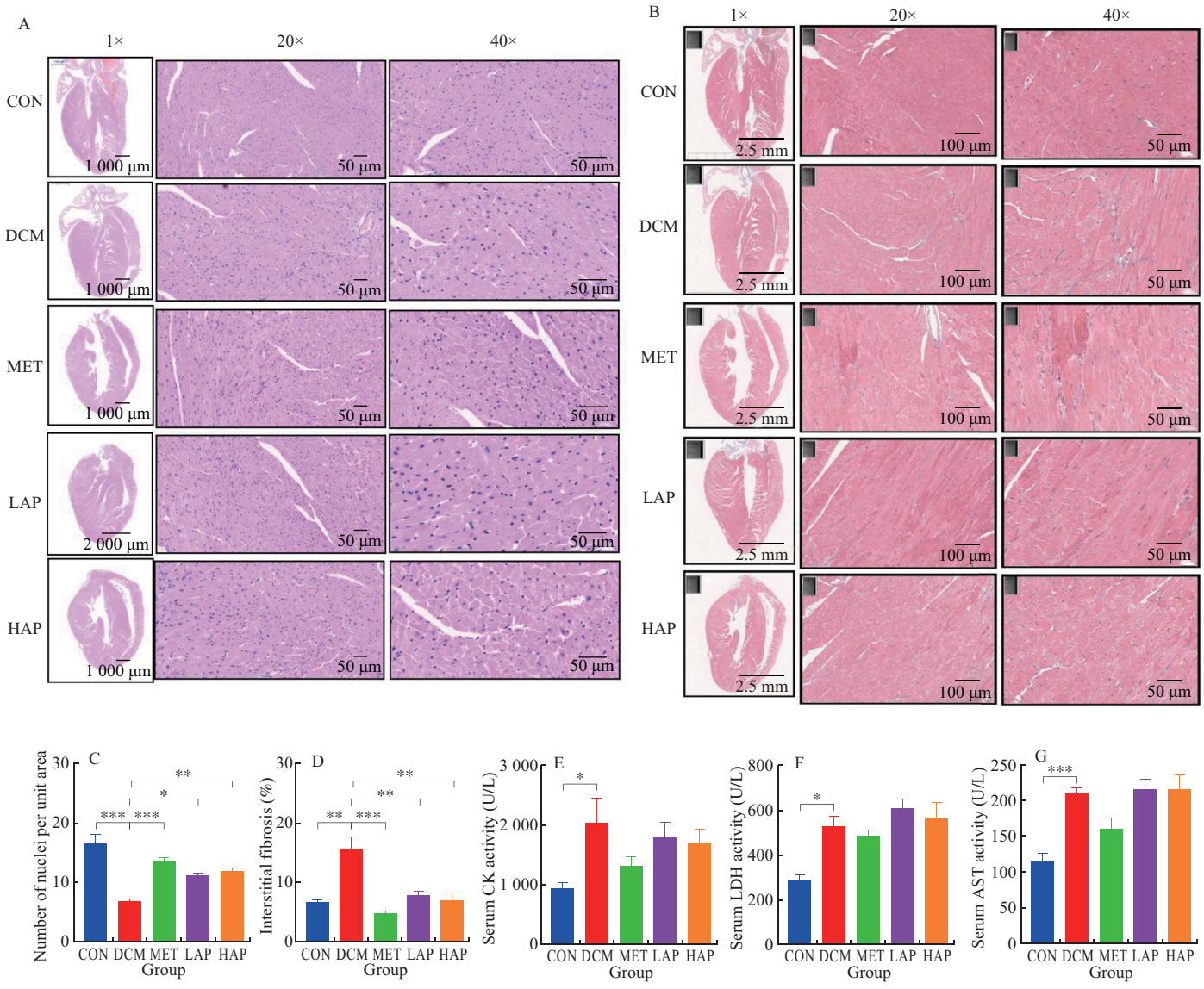

Fig. 4 TYAP relieved cardiac dysfunction in STZ-induced diabetic mice. (A) H&E staining of the heart was photographed at 1×, 20× and 40× magnification; (B) Masson staining of the heart was photographed at 1×, 20× and 40× magnification; (C) the number of nuclei per unit area in H&E staining; (D) the interstitial fibrosis in Masson stain; (E) serum CK activity, (F) serum LDH activity and (G) serum AST activity. The values are presented as the mean ± SEM ($n \geq 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.5 TYAP promoted DNA demethylation in heart of STZ-induced diabetic mice

Previous studies found that dysregulated DNA epigenetic modification metabolism played a key role in the pathological mechanisms of DCM[7,11,29]. In present study, there was no obvious difference in the levels of 5mC among mice heart of all groups, while the levels of 5hmC and the ratio of 5hmC/5mC in DCM group were significantly lower than that in other groups (Figs. 5A-C). In addition, correlation analysis showed that there was a significant positive correlation between 5hmC level and FS (R^2 = 0.544 9, P = 0.035 7), E′/A′ (R^2 = 0.331 5, P = 0.024 7) (Figs. 5I and K), and a significant negative correlation between 5mC levels and E′/A′ (R^2 = 0.451 0, P = 0.006 1) (Fig. 5G).

3.6 TYAP enhanced the protein stability of TET2 by promoting AMPK phosphorylation in heart of STZ-induced diabetic mice

As we all known DNA epigenetic is a dynamic and reversible process consisting mainly of methylation and demethylation. DNA methylation is mainly dependent on the activity of DNMTs and ratio of SAM/SAH[32]. DNA demethylation is mainly dependent on the activity of TETs and mitochondrial function[9,13-14]. It was worth to note that there was no significant difference in the protein levels of DNMTs (including DNMT1, DNMT3A and DNMT3B) and ratio of SAM/SAH in heart of mice among all groups (Figs. 6A, B and E). Although there was no obvious change in protein levels of TET1 and TET3 among all groups, but the protein level of TET2 in DCM group was significantly lower than that in other groups

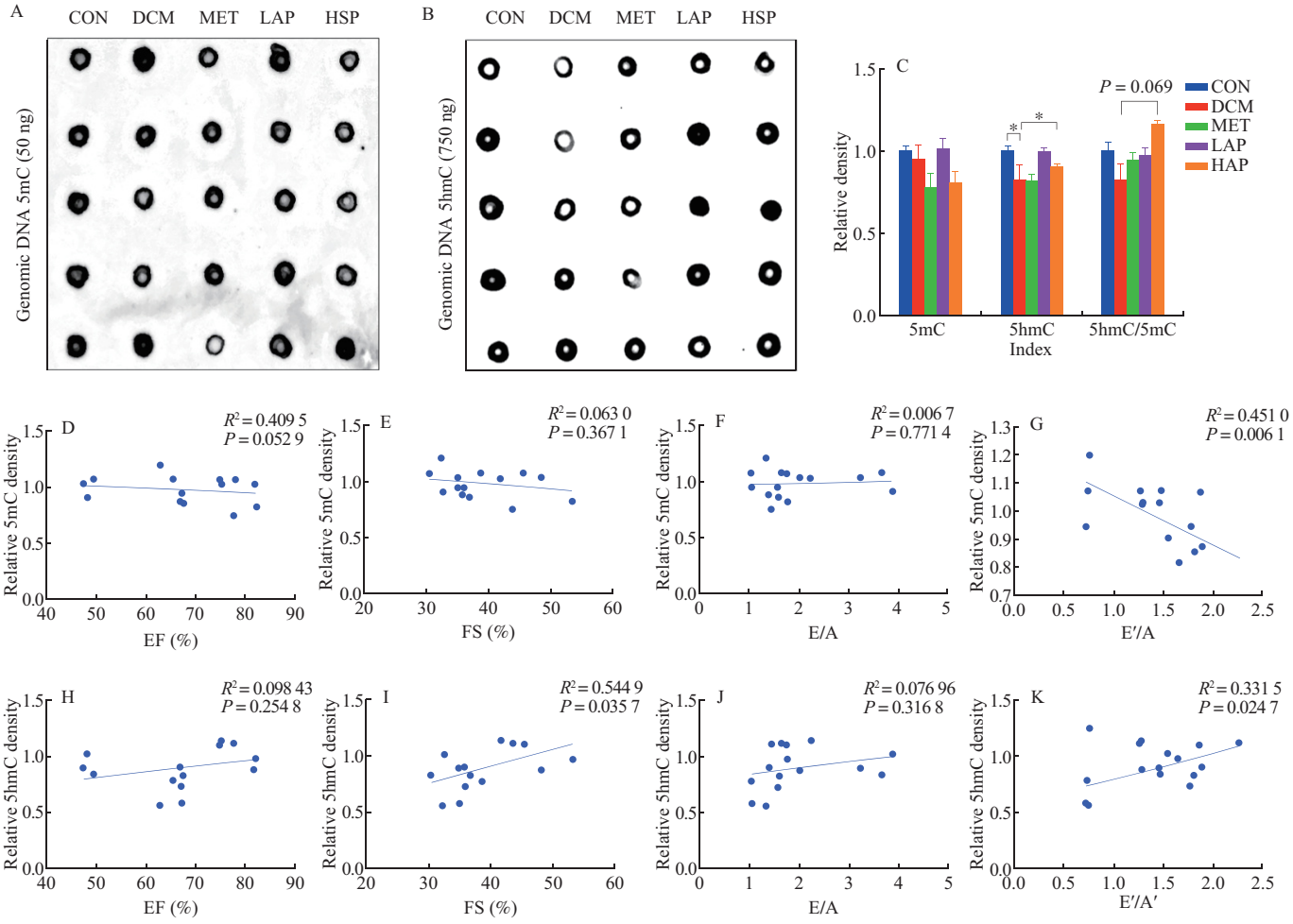

Fig. 5 TYAP promoted DNA demethylation in the heart of STZ-induced diabetic mice.(A) Dot-blotting images of 5mC; (B) dot-blotting images of 5hmC; (C) statistical analysis results of DNA 5mC and 5hmC; (D-K) correlation analysis of DNA 5mC and 5hmC were correlated with cardiac dysfunction. The values are presented as the mean ± SEM ($n \geq 4$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

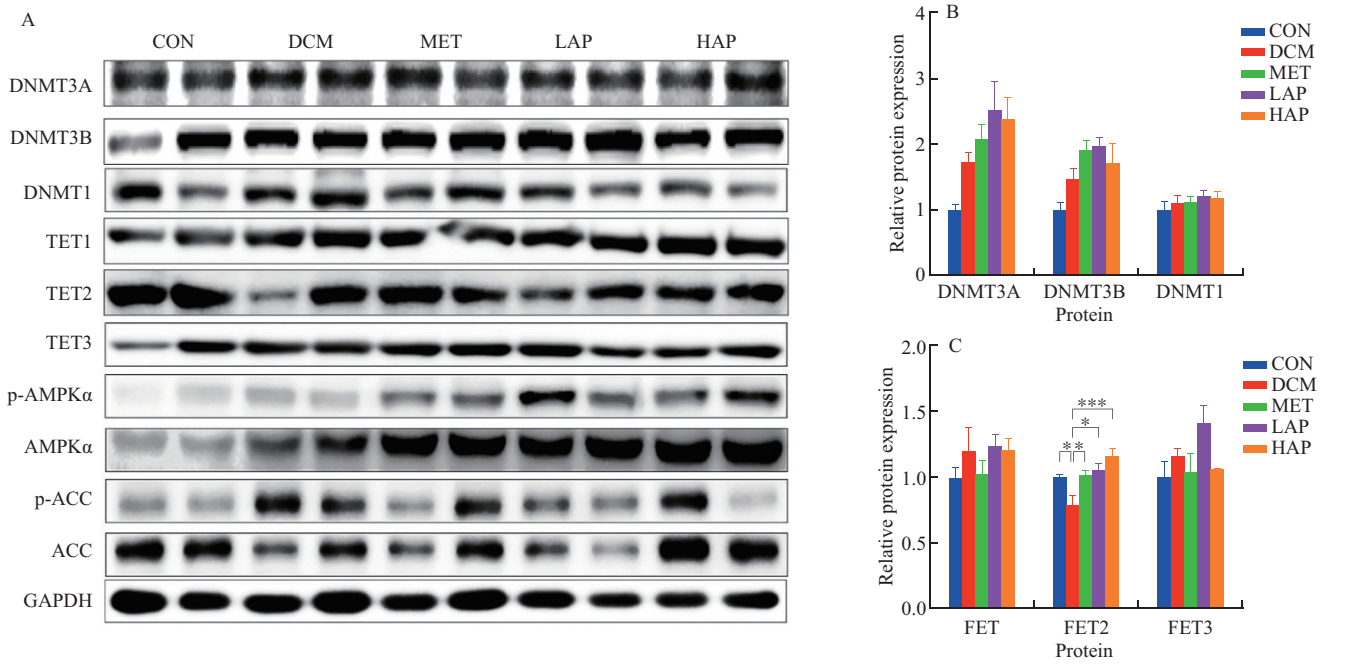

Fig. 6 TYAP enhanced the protein stability of TET2 by promoting AMPK phosphorylation in the heart of STZ-induced diabetic mice. (A) Western blot image of DNMTs, TETs, p-AMPKα, AMPKα, ACC, p-ACC and GAPDH protein levels; (B-D) protein statistical analysis of DNMTs, TETs, p-AMPKα, AMPKα, ACC, p-ACC; (E) the content of SAM, the content of SAH and the ratio of SAM and SAH. The values are presented as the mean ± SEM ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

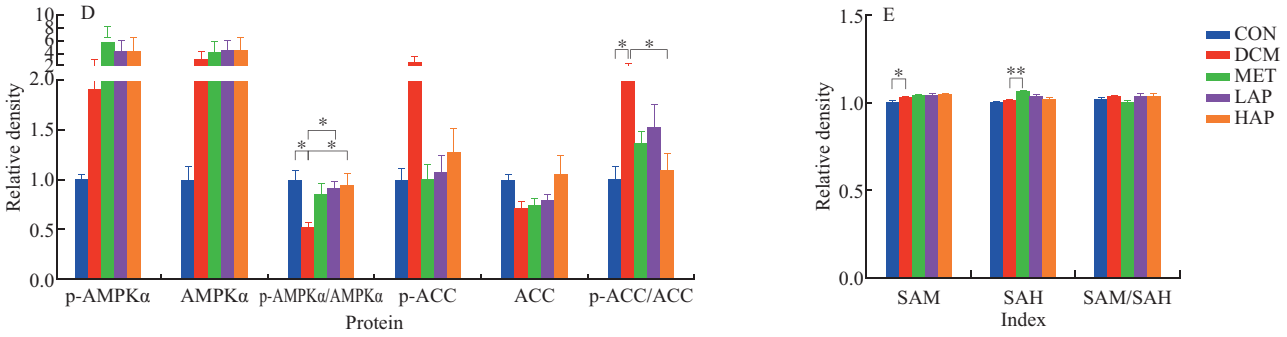

Fig. 6 (*Continued*)

(Figs. 6A and C). As the protein stability of TET2 was regulated by AMPK, we further measured protein levels of p-AMPKα, AMPKα, p-ACC and ACC in mice heart. We found that the ratio of p-AMPKα/AMPKα in DCM group was significantly lower than that in other groups, but the ratio of p-ACC/ACC in DCM group was significantly higher than that in other groups (Figs. 6A and D).

3.7 TYAP enhanced the activity of TETs by promoting the homeostasis of TCA cycle in heart of STZ-induced diabetic mice

The homeostasis of TCA cycle not only plays key regulator role in maintaining cardiac function, but also influences the process of DNA demethylation *via* regulating TET enzymes activity[13,32]. Therefore, we evaluated TCA cycle homeostasis by examining the protein levels of TCA cycle-related enzymes and the concentrations of TCA cycle intermediate metabolites. As shown in Figs. 7A and B, there was no obvious difference in the protein levels of IDH1 and IDH2, SDHB, and MDH2 among groups. Additionally, the protein levels of SDHA and FH were significantly higher in DCM group than these in CON group, and obvious improved by TYAP treatment. Furthermore, we detected the contents of TCA cycle metabolites in mice hearts by LC-MS. It was worth to note that the content of *α*-KG was significantly lower in DCM group than in other groups, while the content of fumaric acid and aconitic acid were significantly higher in DCM group than in other groups (Figs. 7D, F and G). The above results indicated that TYAP promoted TET enzymes activity by improving TCA cycle in heart of diabetic mice.

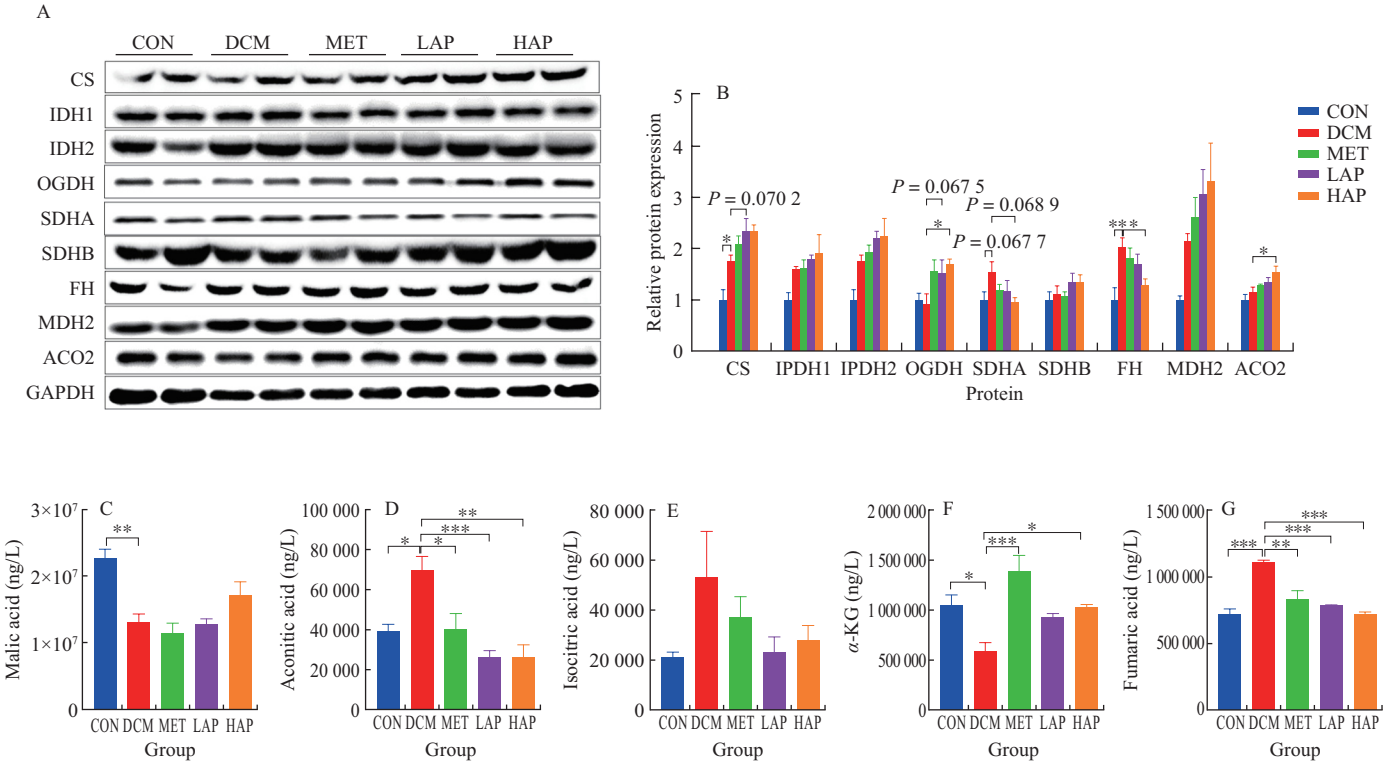

Fig. 7 TYAP promoted the homeostasis of TCA cycle in the heart of STZ-induced diabetic mice. (A) Western blot images of TCA-cycle-related protein levels; (B) protein statistical analysis of TCA-cycle-related protein levels; the contents of (C) malic acid; (D) aconite acid; (E) isocitrate acid; (F) *α*-KG; (G) fumaric acid. The values are presented as the mean ± SEM ($n \geq 4$); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

4. Discussion

Plenty of recent studies have shown that TET-mediated active DNA demethylation plays a critical role in heart development and maintain heart function[11-12,33]. Furthermore, our previously study found that dysregulated DNA hydroxymethylation played a key role in diabetic cardiac dysfunction[7]. However, it still lacks study to examined the levels of 5mC and 5hmC of peripheral blood DNA in DCM patients, and whether could prevent DCM by regulating DNA epigenetic modifications metabolism. In present study, we firstly used a case-control study to investigate the role of DNA epigenetics in the development of DCM, and found that the 5hmC (the main products of DNA demethylation) levels of peripheral blood DNA was significant lower in DCM patients than that age-matched DM patients and health control people (Figs. 1A-D). The results of case-control study suggested that inhibition of DNA demethylation was an important pathogenesis of DCM. To confirm this, we used TYAP (a natural polyphenol extracted from thinned young apple that has been shown to increase the activity of TET2) as the intervention and explored its preventive effect on DCM and molecular mechanism (Fig. 2A).

The chlorogenic acid accounted for about 40% of TYAP polyphenols, as the highest concentration, followed by phlorizin, phloretin and epicatechin accounted for about 14%, 12% and 10%, respectively. Additional, small amounts of anthocyanin polymers, quercetin and rutin were detected[34-35]. The previous studies had showed that 200 or 400 mg/(kg·day) apple polyphenols enhanced anti-inflammatory effects and 125 or 500 mg/(kg·day) apple polyphenols up-regulated the protein expression of AMPK and decreased total cholesterol in liver[35-36]. Therefore, 150 mg/(kg·day) TYAP was used as the low-dose intervention and 300 mg/(kg·day) as the high-dose intervention.

It had been reported that chlorogenic acid, as the main component of TYAP, restored left ventricular systolic and diastolic dysfunction, and reduced myocardial fibrosis caused by DCM in mice[36]. The puerarin had reduced myocardial cell apoptosis and improved mitochondrial function *in vitro*[37], further more, it reduced myocardial fibrosis and prevented heart failure[38]. In our ultrasonic results, it was indicated that TYAP intervention significantly improved the left ventricular systolic and diastolic functions of diabetic mice, and there was a dose dependence relationship on TYAP improving left ventricular function (Fig. 3). Besides, TYAP had a better effect on improving left ventricular function than metformin (Fig. 3). TYAP intervention not only significantly reduced the gap between mouse myocardial cells and the degree of myocardial fibrosis, and similar effect was observed of metformin in H&E staining and Masson staining in mice heart tissue (Fig. 4). In previous research, it had been found that by examining the whole genome map of cardiomyocyte in embryonic, neonatal, adult and hypertrophic mice, TET2 regulated the levels of key cardiac genes transcription and expression through affecting the promoter region DNA methylation levels such as myosin heavy chain 7 (Myh7), thereby regulated cardiac development and function[39]. We had found that pomegranate polyphenols treatment promoted TET2 mediated active DNA demethylation, and significantly increased Myh7b expression to prevent cardiac dysfunction in mice feeding with high-fat diet for 8 weeks[40]. Together all, we supposed that TYAP may promote active DNA demethylation mediated by TET2, maintain normal DNA methylation levels in the promoter regions of genes related to cardiac function, increase the transcriptional expression level of genes, and thus prevent DCM.

To prove the above scientific hypothesis, we addressed the levels of DNA epigenetic modifications in heart of diabetic mice, and found only the level of 5hmC were significant improved in TYAP treatment groups compared with DCM group (Figs. 5A-C). Furthermore, the positive correlation between the levels of 5hmC and ventricular function further suggested that promoting active DNA demethylation is a potential molecular mechanism that TYAP prevents DCM (Figs. 5H-K). As we all known DNA demethylation mainly included passive DNA demethylation and TET-mediated active DNA demethylation[9]. The passive DNA demethylation refers to the dilution of 5mC due to lack of functional DNA methylation maintenance (like donor of methyl groups, DNMTs) during DNA replication. Genetically, DNA methylation is catalyzed by DNMTs, which use SAM as donor of methyl groups, SAH as receptor of methyl groups[9,31]. DNMT1 maintains the methylation during DNA replication and thus known as a maintenance DNA methyltransferase, DNMT3A and DNMT3B establish new methylation patterns to unmodified cytosine and thus known as *de novo* DNA methyltransferase[32]. The ratio of SAM/SAH is used as the indicator for DNA methylation status[41]. In present study, we found that the protein levels of DNMT1/3A/3B and the ratio of SAM/SAH were no obviously difference among groups (Figs. 6A, B, and E). Consistent with our results, previously studies shown that tDNA methylation profile of cardiac cells was no obvious changes in STZ-induced diabetic mice compared with health mice[39]. Furthermore, only the protein level of TET2 was significantly lower in DCM group than in CON group, and significantly improved in TYAP treatment groups (Figs. 6A and C). Taken together, these evidence indicated that TYAP prevented DCM by promoting TET2-mediated active DNA demethylation in STZ-induced diabetic mice.

The process of TET2-mediated active DNA demethylation is mainly dependent on the protein stability of TET2 and its enzyme activity. The protein stability of TET2 was regulated by AMPK, which phosphorylated TET2 at serine 97 and 99 site[14-15]. In present study, we found that the ratio of p-AMPKα/AMPKα was significant improved by TYAP treatment, which means TYAP enhanced the protein stability of TET2 (Fig. 6D). TET2 is a ferrous ion (Fe^{2+}) and α-KG depended on dioxygenase. Our previous studies showed that the activity of TET2 was mainly affected by the levels of α-KG and α-KG inhibitor (succinic acid and fumaric acid), which were the intermediate metabolites of TCA cycle and played key role in maintaining mitochondrial function[13,29,42-43]. In present study, we found that the protein levels of TCA cycle related enzymes (especially OGDH, SDHA, and FH) and content of TCA cycle metabolites (especially α-KG and fumaric acid) were obviously improved in TYAP treatment groups (Fig. 7). Previous studies have proven apple polyphenols to be effective in preventing or treating diabetes and related complications in various ways such as antioxidant, free radical scavenging and anti-inflammatory effects[18,44-45]. The apple polyphenols exhibit various bioactivities are achieved by improving mitochondrial function[20]. It was well known that mitochondrial function plays a critical role in DNA epigenetic modifications metabolism[45]. The above evidence indicated that TYAP promoted TET2-mediated active DNA demethylation by improving AMPK phosphorylation and the homeostasis of TCA cycle in heart of STZ-induced diabetic mice.

Also, there are several limitations in present study to be noted. For the population study, it was hard to prove a causal relationship between DNA demethylation and DCM through case-control study. Therefore, cohort studies and randomized controlled trials are needed to reveal the causal relationship in the future. For the animal study, although our results indicated that TYAP prevent DCM by promoting TET2-mediated active DNA demethylation in the heart of STZ-induced diabetic mice, but the precise molecular mechanisms remain to be determined. TET2 knockout mice are needed to further validate the conclusions of present study. Furthermore, our results only showed that TYAP regulated DNA epigenetic modifications metabolism in global level, the specific genes regulated by methylation or hydroxymethylation involved are still unclear. Genome-wide DNA methylation or hydroxymethylation sequencing are needed to screen out the specific genes regulated in future studies.

5. Conclusion

In conclusion, our results demonstrated that active DNA demethylation in peripheral blood was inhibited in DCM patients compared with DM and health population. Furthermore, as a natural polyphenol extracted from thinned young apple, TYAP had been shown to increase the activity of TET2, effectively preventing the ventricular dysfunction and cardiomyocyte disarray by promoting TET2-mediated active DNA demethylation in heart of STZ-induced diabetic mice. Overall, this study provides a new avenue to prevent DCM that is targeting TET2-mediated active DNA demethylation and indicates that increasing apple polyphenols intake prevents DCM.

Ethics approval and consent to participate

The Case-control study was approved by the Ethics Committee of Qingdao University School of Medicine (QDU-HEC-2022282). The animal experiment procedures adhered to Qingdao University Guide for the care and use of Laboratory Animals and approved by Ethics Committee Medical College of Qingdao University (QDU-AEC-2022462).

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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