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Sodium–glucose co-transporter 2 inhibition with empagliflozin improves cardiac function in non-diabetic rats with left ventricular dysfunction after myocardial infarction

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Aims

Sodium–glucose co-transporter 2 (SGLT2) inhibition reduces heart failure hospitalizations in patients with diabetes, irrespective of glycaemic control. We examined the effect of SGLT2 inhibition with empagliflozin (EMPA) on cardiac function in non-diabetic rats with left ventricular (LV) dysfunction after myocardial infarction (MI).

Methods and results

Non-diabetic male Sprague–Dawley rats underwent permanent coronary artery ligation to induce MI, or sham surgery. Rats received chow containing EMPA that resulted in an average daily intake of 30 mg/kg/day or control chow, starting before surgery (EMPA-early) or 2 weeks after surgery (EMPA-late). Cardiac function was assessed using echocardiography and histological and molecular markers of cardiac remodelling and metabolism were assessed in the left ventricle. Renal function was assessed in metabolic cages. EMPA increased urine production by two-fold without affecting creatinine clearance and serum electrolytes. EMPA did not influence MI size, but LV ejection fraction (LVEF) was significantly higher in the EMPA-early and EMPA-late treated MI groups compared to the MI group treated with vehicle (LVEF 54%, 52% and 43%, respectively, all $P < 0.05$). EMPA also attenuated cardiomyocyte hypertrophy, diminished interstitial fibrosis and reduced myocardial oxidative stress. EMPA treatment reduced mitochondrial DNA damage and stimulated mitochondrial biogenesis, which was associated with the normalization of myocardial uptake and oxidation of glucose and fatty acids. EMPA increased circulating ketone levels as well as myocardial expression of the ketone body transporter and two critical ketogenic enzymes, indicating that myocardial utilization of ketone bodies was increased. Together these metabolic changes were associated with an increase in cardiac ATP production.

Conclusion

Empagliflozin favourably affects cardiac function and remodelling in non-diabetic rats with LV dysfunction after MI, associated with substantial improvements in cardiac metabolism and cardiac ATP production. Importantly, it did so without renal adverse effects. Our data suggest that EMPA might be of benefit in heart failure patients without diabetes.

Keywords

Diabetes • Heart failure • Metabolism • Mitochondria • Renal function • Remodelling

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Introduction

Type 2 diabetes mellitus (T2DM) and heart failure (HF) commonly coexist.¹ Patients with T2DM have a two-fold increased risk of developing HF during their lifetime,^{2,3} and are also twice as likely to be hospitalized for HF.^{4–6}

Cardiovascular events and HF hospitalizations can be reduced by adequate glycaemic control in patients with T2DM,⁷ but once HF develops the therapeutic options become limited.^{8–10} Currently, only metformin is considered safe in HF patients because other oral anti-diabetic drugs have suspected or established safety concerns in this population.¹ This is not the case for sodium–glucose co-transporter 2 inhibitors (SGLT2i), a new class of anti-diabetic drugs that have been shown to markedly reduce cardiovascular events and HF hospitalizations in patients with T2DM. SGLT2i are not only safe and well tolerated in patients with HF but also reduce the incidence of HF hospitalizations, both in subgroups of patients with and without HF at baseline.^{11,12}

SGLT2 is a sodium-dependent glucose transporter expressed in the proximal tubule of the kidney and is responsible for 90% of the renal reabsorption of filtered glucose. Accordingly, SGLT2i induce urinary glucose and sodium excretion and promote diuresis.¹³ SGLT2i have relatively few side effects and rarely cause symptomatic hypoglycaemia, even when given to non-diabetic patients.¹⁴ SGLT2i have relatively modest effects on glycaemic control, suggesting that alternative mechanisms may be responsible for the reductions in HF events, which may also apply to non-diabetic patients. The effects of SGLT2i on diuresis and HF hospitalizations without significant side effects had prompted the hypothesis that SGLT2i could also be of benefit in HF patients, with or without T2DM.

While this hypothesis is currently under investigation in several clinical trials,^{15,16} the effect of SGLT2 inhibition on outcomes in non-diabetic HF patients remains unclear at this point.^{17,18} We sought to determine the effect of the SGLT2i empagliflozin (EMPA) on cardiac function and remodelling after myocardial infarction (MI).

Methods

A detail description of methods is available in the online supplementary *Methods S1*.

Experimental protocol

The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen (IvD number: 16487-02-001). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. We followed ARRIVE guidelines when reporting this study.¹⁹ Non-diabetic male Sprague–Dawley rats (Envigo, The Netherlands) were randomized to treatment with chow containing EMPA or control chow, starting either 2 days before surgery (early) or 2 weeks after surgery (late). Treatment allocation in the late groups was stratified according to left ventricular ejection fraction (LVEF) 2 weeks post-surgery to ensure that baseline cardiac function is similar in the EMPA and the vehicle groups. After 10 weeks of treatment, rats were anaesthetized,

blood was drawn, and the hearts were rapidly excised for further analysis. Rats with an infarct size of <15% were excluded from the analysis as these small infarcts are haemodynamically fully compensated.²⁰

Myocardial infarction surgery

Rats were randomized to permanent ligation of the left anterior descending coronary artery or sham surgery under isoflurane (2.5%) inhalation anaesthesia, as previously described.²¹

Investigational drug

Empagliflozin (BI 10773) was kindly supplied by Boehringer Ingelheim, Germany. EMPA (BI 10773) was mixed with standard rat chow (R/M-H V1534-70, Ssniff, Germany) in a final concentration of 200 mg/kg intended to reach an average dose of 30 mg/kg/day.

Echocardiography

Two weeks after surgery and 1 week before termination, the M-mode and two-dimensional echocardiography was performed using a Vivid 7 echo machine (GE Healthcare, Milwaukee, WI, USA) equipped with a 10 MHz phased array linear transducer for serially assessment of cardiac structure and function, as previously described.^{21,22}

Invasive haemodynamic measurements

Prior to sacrifice, invasive haemodynamics were analysed by aortic and left ventricular (LV) catheterization, as previously described.²¹ The data were acquired using a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO, USA) and analysed with a LabChart 8 software.

Infarct size, cardiomyocyte size and interstitial fibrosis measurement

For immunohistochemical analysis, the mid-papillary slice of the left ventricle was fixed in 4% formaldehyde and paraffin-embedded. Masson's trichrome staining was performed to evaluate the infarct size and the extent of interstitial fibrosis. Furthermore, to determine cardiomyocyte size, sections were stained with FITC-labelled wheat germ agglutinin, as previously described.^{21,22}

Metabolic cage

Two weeks before termination, rats were placed in metabolic cages to monitor 24 h water and food intake and 24 h urine collection, as previously described.²³

Blood and urine measurements

Blood samples were obtained via tail vein at regular intervals under isoflurane anaesthesia to monitor blood glucose and haematocrit levels. At sacrifice, 8 mL of blood was drawn from the abdominal aorta (either anticoagulated with EDTA or sodium heparin), and urine was collected directly from bladder.

Mitochondrial DNA (mtDNA)-to-nuclear DNA (nDNA) ratio and mtDNA damage

Total DNA including mtDNA was extracted from the non-infarcted left ventricle using Nucleospin[®] Tissue XS (Macherey-Nagel GmBH&Co. KG, Düren, Germany). mtDNA-to-nDNA ratio was determined by quantitative real-time polymerase chain reaction (qRT-PCR), as described previously.²⁴ Expression of mitochondrial genes were corrected for nuclear gene expressions values, and the calculated values were expressed relative to the control group per experiment. To determine DNA damage (lesions/10 kb), the D-loop mitochondrial genomic region was amplified by a semi-long-run qRT-PCR, as described before.²⁴ Primer sequences are listed in the online supplementary Table S1.

Quantitative real-time polymerase chain reaction

RNA was extracted from the non-infarcted left ventricle using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and QuantiTect RT kit (Qiagen) was then used to make cDNA, following manufacturer's instructions, as previously described.²⁵ 36B4 reference gene was used to correct all measured mRNA expression. Primer sequences can be found in the online supplementary Table S1.

Advanced oxidation protein products measurements

The advanced oxidation protein product (AOPP) assay was performed using the AOPP Assay Kit from Abcam (#ab242295, Cambridge, UK) according to the manufacturer's instructions. Results were then normalized by protein concentrations of each test.

ATP measurements

The ATP assay was performed using the ATP Assay Kit (colorimetric/fluorometric) from Abcam (#ab83355, Cambridge, UK) according to the manufacturer's instructions. Results were then normalized by protein concentrations of each test.

Pyruvate dehydrogenase activity

According to manufacturer's instruction, we measured the activity of pyruvate dehydrogenase (PDH) using the assay kit from Sigma MAK183 and a spectrophotometric multiwell plate reader (Synergy H1, BioTek).

Insulin and glucagon measurements

Circulating hormones were measured by a rat ultrasensitive insulin ELISA kit (80-INSRT-E01, ALPCO) and glucagon ELISA (48-GLUHU-E01, ALPCO) according to the manufacturer's instructions.

Statistical analysis

Data are presented as means \pm standard errors of the mean. To compare normally distributed parameters, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was

used. When data were not normally distributed, a non-parametric Kruskal–Wallis test followed by a Mann–Whitney U test with correction for multiple comparisons was used. To compare EMPA and vehicle treatment independent of treatment allocation, an independent *t*-test or a Mann–Whitney U test was used, where appropriate. Wilcoxon signed rank test was used to evaluate LVEF post-MI vs before termination. Differences were considered significant at $P < 0.05$. IBM SPSS statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA) was used to perform all statistical analysis.

Results

A total of 140 rats were randomized to MI or sham surgery, 47 rats died during the surgical procedure. Overall mortality in the MI groups was 41%. All rats died from ventricular fibrillation within 60 min of left anterior descending coronary artery ligation. No mortality was observed during the subsequent stages of the study and no mortality was observed in sham-operated rats throughout the study. There were no statistically significant differences in mortality between the MI-vehicle vs. the MI-EMPA-early or the MI-EMPA-late groups (43% vs. 40% and 40%, respectively). In total, 20 rats with infarct sizes of $< 15\%$ were excluded from the analysis (8 rats in the MI-vehicle group, 5 rats in the MI-EMPA-early group and 7 rats in MI-EMPA-late group), leaving a total of 73 rats for the current analysis. The final group sizes were 8 for the sham-vehicle group, 19 for the sham-EMPA group, 22 for the MI-vehicle group, 13 for the MI-EMPA-early group, and 11 for the MI-EMPA-late group.

Efficacy and safety of empagliflozin in non-diabetic rats with left ventricular dysfunction after myocardial infarction

Efficacy of empagliflozin

Daily food intake was comparable between EMPA and vehicle-treated groups and the average daily dose of EMPA was 30 mg/kg of body weight/day (online supplementary Figure S1A). As expected, EMPA increased urinary glucose (Figure 1A) and sodium excretion (Figure 1B), resulting in a two-fold increase in urine production (Figure 1C). The increase in urinary glucose excretion was associated with a substantial reduction in body weight in the EMPA-treated sham and MI groups (online supplementary Figure S1C).

Safety

The increase in diuresis after EMPA treatment was compensated by a proportionate increase in fluid intake (online supplementary Figure S1B) and did not affect haematocrit (online supplementary Figure S2A) or renal function (Figure 1D), indicating that EMPA did not cause excessive plasma contraction. In addition, EMPA did not cause significant changes in plasma glucose, sodium, or potassium levels (online supplementary Figure S2B–D).

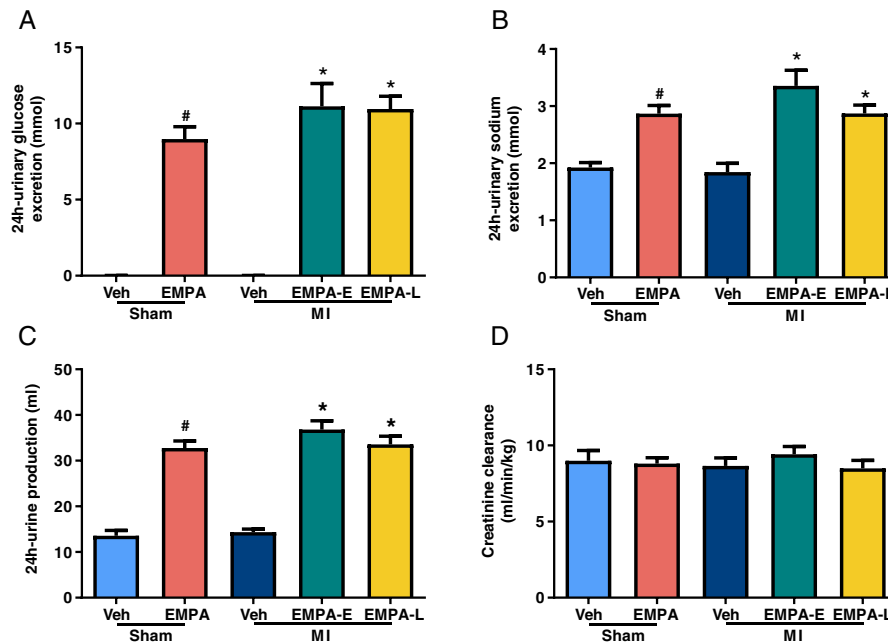


Figure 1 Efficacy and safety of empagliflozin (EMPA) in non-diabetic rats with left ventricular dysfunction after myocardial infarction (MI). (A) 24 h urinary glucose excretion. (B) 24 h urinary sodium excretion. (C) 24 h urine production. (D) Creatinine clearance of all groups. EMPA-E, EMPA-early; EMPA-L, EMPA-late; Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; # $P < 0.05$ vs. Sham-Veh.

Effect of empagliflozin on blood pressure and cardiac function

The average LV infarct size was 33% and did not differ among the MI, MI-EMPA-early and MI-EMPA-late groups (Figure 2A and 2B). Systolic and diastolic blood pressure was also not different among groups (online supplementary Table S2). As expected, MI resulted in significant LV dilatation (Figure 2C) and a reduction in LVEF (Figure 2D). LVEF was significantly higher in both MI-EMPA-treated groups compared to the MI-vehicle group (Figure 2D). A pooled analysis of longitudinal changes in LVEF revealed that EMPA prevented the progressive deterioration of cardiac function that occurs after MI (Figure 2E). Other relevant echocardiographic parameters are depicted in the online supplementary Table S3. There was no significant between-group difference in LV filling pressures (online supplementary Table S2).

Effect of empagliflozin on cardiac histology and molecular markers for remodelling and fibrosis

A large MI invariably causes pathological remodelling of the non-infarcted myocardium, which contributes to further deterioration of cardiac performance.^{26–28}

Marked cardiac hypertrophy was observed after MI as reflected by a 10% increase in ventricular mass (Figure 2F) and an 81% increase in cardiomyocyte cross-sectional area (Figure 3A and 3B). Both early and late treatment with EMPA attenuated the increase in

LV mass (Figure 2F) and diminished cardiomyocyte cross-sectional area compared to the vehicle-treated MI group (Figure 3A and 3B). Myocardial fibrosis was increased three-fold in the non-infarcted left ventricle, and this was also markedly attenuated by EMPA treatment (Figure 3B and 3C). The reductions in fibrosis after EMPA treatment were accompanied by similar reductions in the expression of the fibrosis markers collagen 1 and procollagen (Figure 3E).

Increased myocardial expression of atrial natriuretic peptide (ANP) and an increase in the relative expression of foetal (β -MHC) over adult (α -MHC) myocin heavy chain isoform (i.e. β -MHC/ α -MHC ratio) are generally considered as robust markers for the activation of the cardiac foetal gene programme.²⁹ Myocardial ANP expression was increased by six-fold in the vehicle-treated MI group compared to the sham groups. Both early and late treatment with EMPA reduced cardiac ANP expression by 50%. Similarly, the increase in β -MHC/ α -MHC ratio observed in the MI group was normalized to sham levels after EMPA treatment (Figure 3D).

Effects of empagliflozin on oxidative stress

To investigate the effect of EMPA on myocardial oxidative stress, we determined the advanced oxidation protein product (AOPP) level and the expression level of the superoxide generating enzyme NOX2. AOPP was higher in the heart of rats in the MI-vehicle group than in the sham-vehicle group, and was suppressed by EMPA

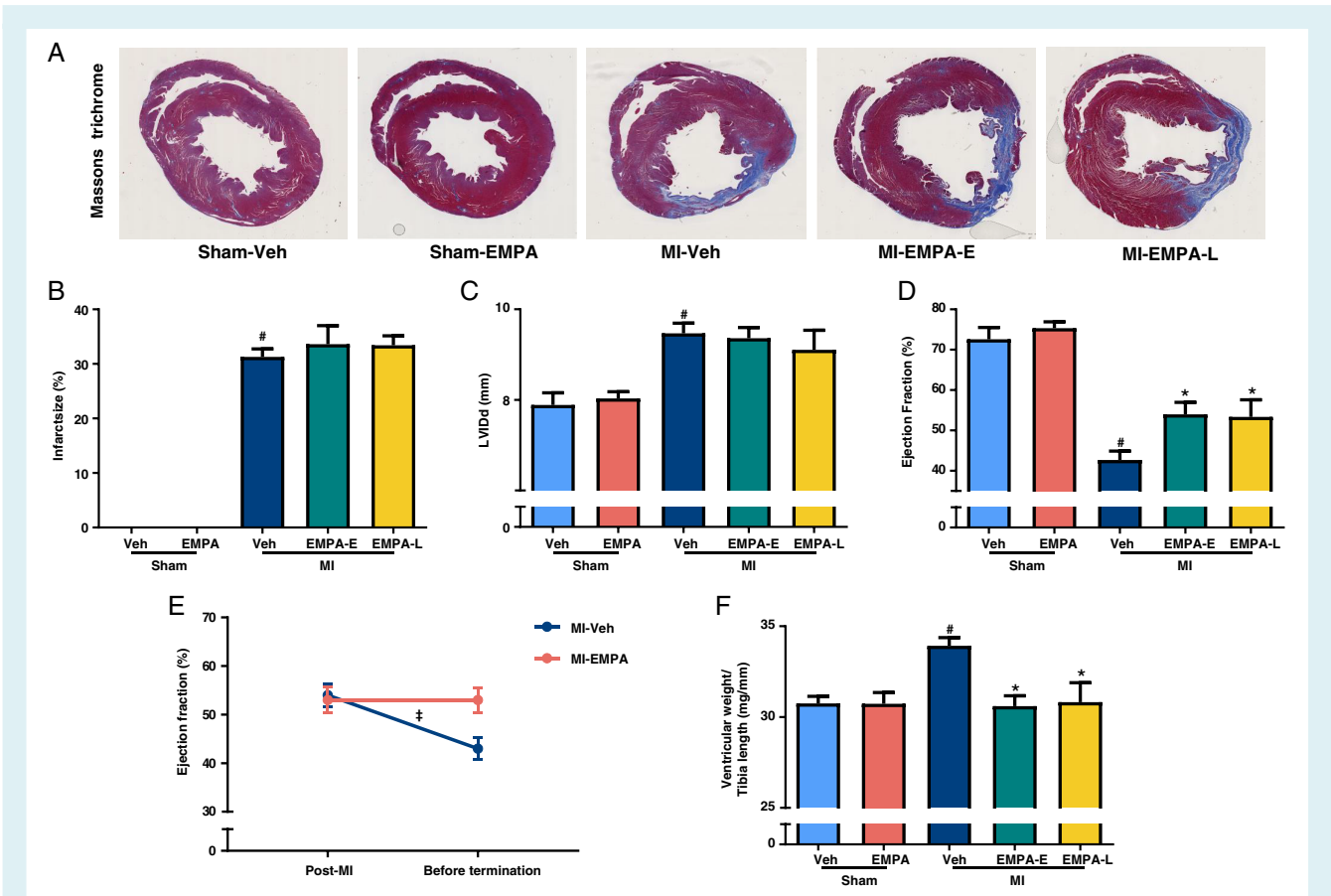


Figure 2 Effect of empagliflozin (EMPA) on cardiac function in non-diabetic rats with left ventricular dysfunction after myocardial infarction (MI). (A) Representative left ventricular sections stained with Masson's trichrome. (B) Quantification of infarct size from Masson's trichrome stained section. (C) Left ventricular internal dimensions in diastole (LVIDd). (D) Left ventricular ejection fraction. (E) Longitudinal change of left ventricular ejection fraction post-MI and before termination. (F) Ratio of ventricular weight to tibia length. EMPA-E, EMPA-early; EMPA-L, EMPA-late. Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; $\#P < 0.05$ vs. Sham-Veh; $\ddagger P < 0.05$ vs MI-Veh post-MI.

in both early and late treatment (Figure 4A). Similarly, expression of NOX2 was up-regulated in the MI-vehicle group, but was attenuated in both the MI-EMPA-early and MI-EMPA-late groups (Figure 4A).

Effects of empagliflozin on mitochondrial biogenesis and ATP production

Detailed cardiac and renal analysis of diabetic animal models has revealed that SGLT2i affect mitochondrial morphology and density.^{30,31} Mitochondrial content and respiratory capacity are also significantly reduced in HF, which is tied to diminished expression of the transcription factor PGC-1 α .³² To determine whether the favourable effects of EMPA on cardiac remodelling were associated with similar recovery of mitochondrial biogenesis, mitochondrial content was quantified by normalizing mtDNA to nDNA. We also performed a semi-long-run PCR to detect mtDNA damage. As expected, mtDNA damage was also increased after MI (Figure 4B). In addition, mtDNA/nDNA ratio was markedly

reduced after MI (Figure 4C), accompanied by similar reductions in PGC-1 α expression (Figure 4C). EMPA normalized PGC-1 α expression and partially recovered mtDNA/nDNA (Figure 4A) and mtDNA damage (Figure 4B).

To verify whether the effects of EMPA on mtDNA damage and the increase in mtDNA/nDNA were associated with increased ATP production, we determined the cardiac ATP levels in our study. ATP levels were significantly reduced in the MI-vehicle group and, interestingly, were significantly restored in the MI-EMPA-early group, and there was a trend towards increased ATP levels in the MI-EMPA-late group ($P = 0.08$) (Figure 4D).

Effects of empagliflozin on myocardial glucose and fatty acid metabolism

Heart failure is accompanied by profound changes in myocardial utilization of carbon-based fuels.³³ During HF development, cardiac substrate preference first shifts from fatty acids to glucose as the primary fuel source, while the later stages of HF are

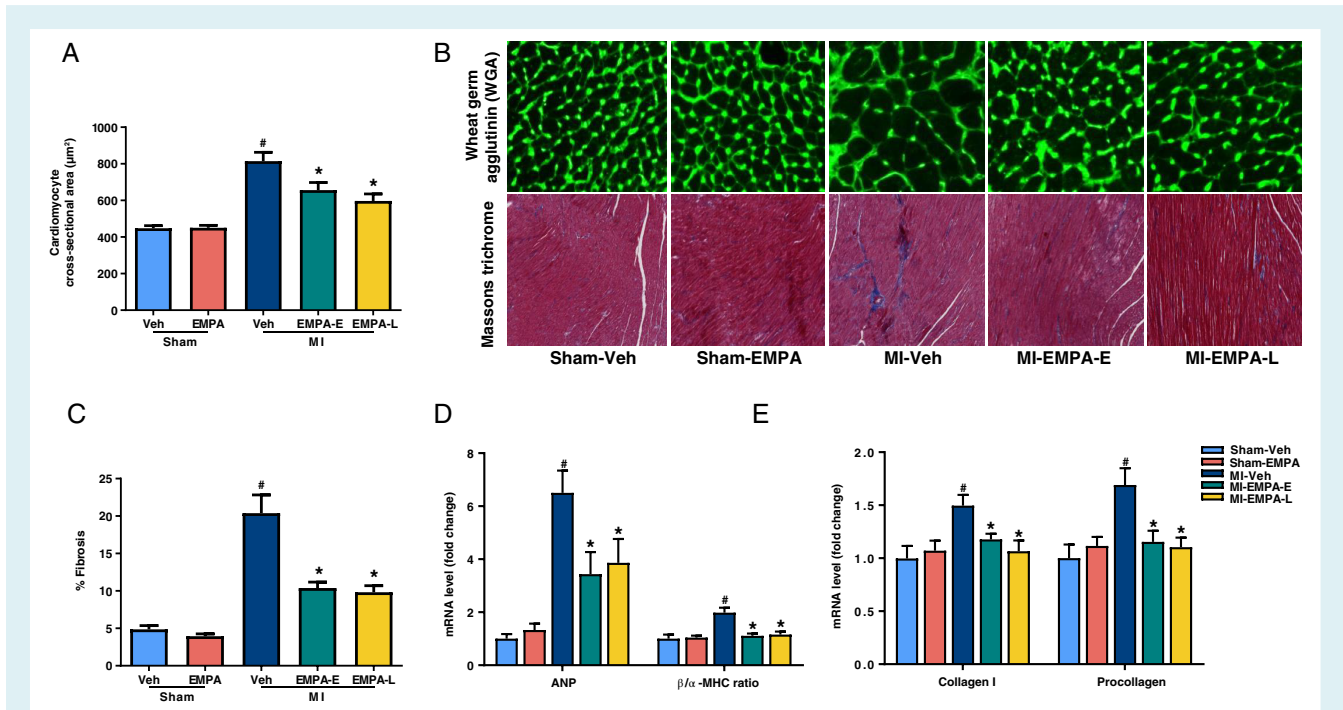


Figure 3 Effect of empagliflozin (EMPA) on pathological cardiac remodelling and fibrosis in non-diabetic rats with left ventricular dysfunction after myocardial infarction (MI). (A) Quantification of cardiomyocyte cross-sectional area from wheat germ agglutinin (WGA) stained section. (B) Representative left ventricular sections stained with WGA and Masson's trichrome staining to assess cardiomyocyte hypertrophy and fibrosis. (C) Quantification of fibrosis in the non-infarcted left ventricle from Masson's trichrome stained section. (D,E) Measurement of mRNA levels to assess molecular markers for remodelling and fibrosis, respectively, normalized to 36B4. EMPA-E, EMPA-early; EMPA-L, EMPA-late; Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; # $P < 0.05$ vs. Sham-Veh.

also associated with disruption of myocardial glucose uptake and utilization.³³ Molecular signatures for impaired substrate utilization are, among others, down-regulation of the fatty acid transporter carnitine palmitoyltransferase 1- α (CPT1- α), down-regulation of the glucose transporter type 4 (GLUT4) and inhibition of the pyruvate dehydrogenase complex through up-regulation of pyruvate dehydrogenase kinase 4 (PDK4).³³ Pyruvate dehydrogenase is required for carbohydrate intermediates to enter the Krebs cycle and PDK4 inhibits this enzyme complex.³³

As expected, CPT1- α , GLUT4, PGC1- α levels and PDH activity were reduced and PDK4 expression was increased in cardiac tissue of the MI-vehicle group compared to the sham-vehicle group. Interestingly, EMPA treatment restored the expression of all these markers to sham levels (Figure 5A, 5B and 5D), suggesting that EMPA restores myocardial substrate metabolism.

Effects of empagliflozin on ketone metabolism

The disruption of glucose and fatty acid oxidation causes the failing heart to increasingly rely on ketone bodies as a fuel source.^{34,35} EMPA increases circulating ketone levels and indirect evidence suggests that EMPA promotes ketone utilization in patients with diabetes.³⁶ The beneficial effects of EMPA on cardiac performance

could therefore be explained by increased myocardial ketone utilization.^{37,38}

Hepatic ketogenesis is induced by reductions in the systemic insulin/glucagon ratio and EMPA decreased insulin-to-glucagon ratio (Figure 5C). Consistent with these observations, EMPA treatment increased both circulating ketone levels and urinary ketone excretion in sham and MI groups (Figure 6A and 6B). To determine whether the increases in circulating ketone levels was also associated with changes in the myocardial capacity to utilize ketone bodies, we performed a detailed analysis of three critical proteins involved in myocardial ketolysis, namely the ketone body transporter monocarboxylate transporter 1 (MCT1), ketogenic enzyme β -hydroxy butyrate dehydrogenase (BDH1) and succinyl-CoA:3-ketoacid CoA transferase (SCOT). The expression of the MCT1 and BDH1 gene and the protein expression of SCOT were all significantly increased in the MI-vehicle group. Furthermore, the expression was further increased in both MI-EMPA-early and MI-EMPA-late groups (Figure 6C and 6D). These findings suggest that ketone bioavailability and cardiac ketone utilization are increased by EMPA.

Discussion

In the present study in which we treated non-diabetic rats with LV dysfunction after a large MI with the SGLT2i EMPA for 10 weeks,

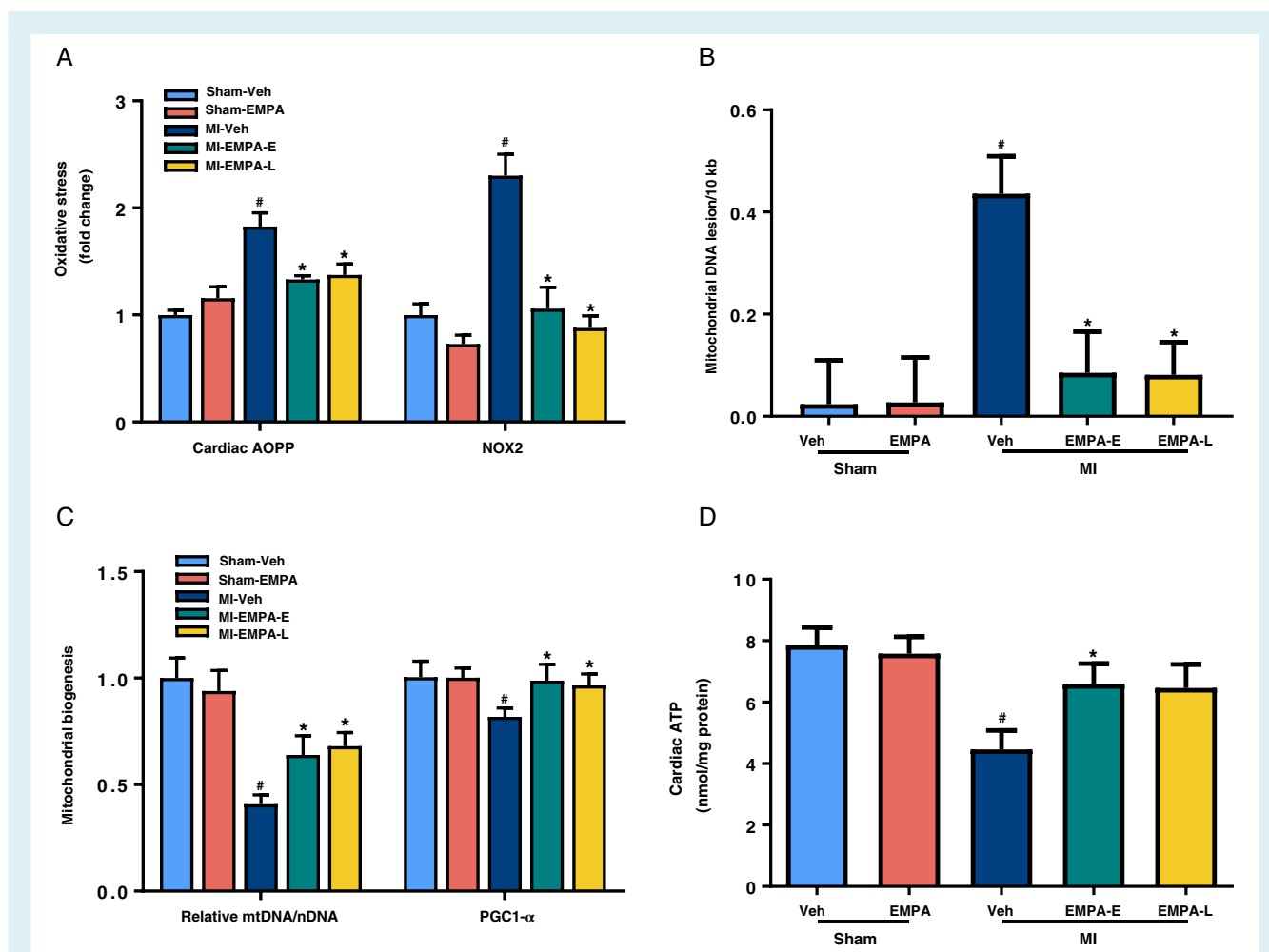


Figure 4 Effect of empagliflozin (EMPA) on oxidative stress, mitochondrial biogenesis and cardiac ATP levels. (A) Cardiac advanced oxidation protein products and measurement of mRNA levels to assess oxidative stress. (B) Semi-long-run polymerase chain reaction was performed using primers specific for mitochondrial DNA fragments in the mitochondrial D-loop region to determine mitochondrial DNA damage. (C) Mitochondrial DNA (CYTB)-to-nuclear DNA (TRPM-2) ratio and mRNA levels to assess mitochondrial biogenesis. (D) Cardiac ATP levels. EMPA-E, EMPA-early; EMPA-L, EMPA-late; MI, myocardial infarction; Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; # $P < 0.05$ vs. Sham-Veh.

diuresis was markedly increased without affecting kidney function, serum glucose and electrolyte levels. EMPA did not influence MI size, but it did improve cardiac function and attenuated pathological cardiomyocyte hypertrophy and cardiac fibrosis. The beneficial effects of EMPA on cardiac function were associated with favourable effects on cardiac metabolism, including reduction of mitochondrial DNA damage and oxidative stress, activation of mitochondrial biogenesis, and the restoration of cardiac glucose and fatty acid oxidation. Furthermore, EMPA promoted the bioavailability of ketones as well as the cardiac capacity to utilize ketone bodies as a fuel source. These findings provide robust evidence that EMPA improves cardiac performance in non-diabetic failing hearts, and provide further mechanistic insights and a rationale for the clinical trials that are underway.

SGLT2i have emerged as oral anti-diabetic drugs that reduce cardiovascular events and HF hospitalizations in patients

with diabetes.^{11,12,39} Pre-specified secondary analysis of the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME) revealed that EMPA reduced new-onset HF and that the reduction in HF hospitalizations was consistent among several subgroups, including patients with HF at baseline.¹¹ In the Canagliflozin Cardiovascular Assessment Study (CANVAS), the SGLT2i canagliflozin also reduced HF hospitalizations compared to placebo and the reductions in HF hospitalizations were more pronounced in patients with a history of HF at baseline.¹² It has to be noted that the history of HF was not robustly adjudicated and that the results of these secondary analyses should be considered hypothesis generating. The validity of this hypothesis is currently being tested in dedicated cardiovascular outcome trials in HF patients with reduced and preserved ejection fraction (NCT03057977,

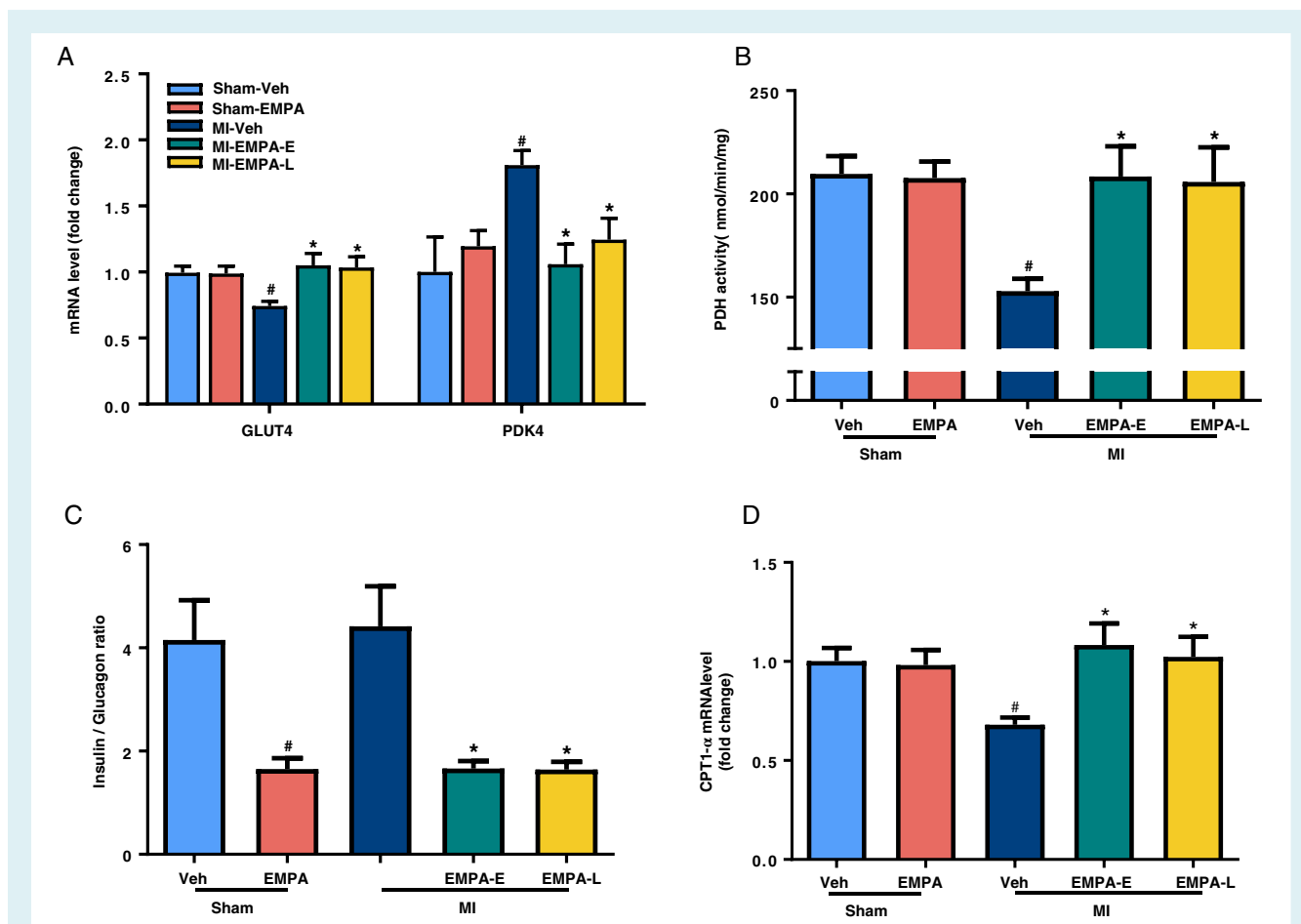


Figure 5 Effect of empagliflozin (EMPA) on glucose metabolism, insulin/glucagon ratio and fatty acid metabolism. (A) Measurement of mRNA levels to assess cardiac glucose metabolism. (B) Pyruvate dehydrogenase (PDH) activity. (C) Insulin/glucagon ratio. (D) Measurement of mRNA levels to assess fatty acid metabolism. CPT1- α , carnitine palmitoyltransferase 1- α ; EMPA, empagliflozin; EMPA-E, EMPA-early; EMPA-L, EMPA-late; MI, myocardial infarction; Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; # $P < 0.05$ vs. Sham-Veh.

NCT03057951, NCT03036124, NCT03619213), which are expected to be completed in 2019–2022.

Interestingly, these trials have been launched in the absence of robust evidence that SGLT2i affect cardiac performance in the absence of diabetes. Indeed, most mechanistic studies have been performed in the context of diabetes. Byrne *et al.*⁴⁰ reported that EMPA treatment prevented the progressive deterioration of cardiac dysfunction in a model of transverse aortic constriction surgery *in vivo* and *ex vivo*. Another study reported that EMPA reduced cardiac hypertrophy and fibrosis in a rat model of metabolic syndrome with pre-diabetes.⁴¹ Moreover, EMPA was also shown to improve diastolic function and ameliorate cardiac hypertrophy and fibrosis in female db/db mice.⁴² Evidence for clinical translation of these findings was recently provided by the EMPA-HEART CardioliNK-6 study, which demonstrated that 6 months of treatment with EMPA resulted in reduced cardiac mass and increased LVEF (Verma S., unpublished data).

In the non-diabetic context, a study of Lee *et al.*⁴³ demonstrated that the SGLT2i dapagliflozin and phlorizin attenuated

oxidative stress after MI in non-diabetic male Wistar rats. They also observed that SGLT2i did not reduce the infarct sizes, however it did attenuate cardiac fibrosis.⁴³ Our data extend this observation as we demonstrate a reduction in fibrosis and oxidative damage to mtDNA. In addition, we have now provided evidence that EMPA also attenuates total myocardial oxidative stress as evidenced by reductions in AOPP and NOX2 expression (Figure 4A).

The mechanisms responsible for the beneficial effects of SGLT2i on HF outcomes are a matter of intense speculation.^{15,18} The most obvious beneficial mechanisms could be derived from the fact that SGLT2i are potent proximal tubule diuretics. The diuretic effects of EMPA were obvious from our study and, in contrast to other diuretics, EMPA did not affect renal function, and plasma levels of glucose and electrolytes remained normal. These findings are consistent with the reno-protective effects observed in patient with diabetes.^{44,45} Of note, while loop diuretics are commonly used to reduce symptoms in patients with HF, there are no data to support a prognostic benefit of using diuretics in this population.⁸ Another

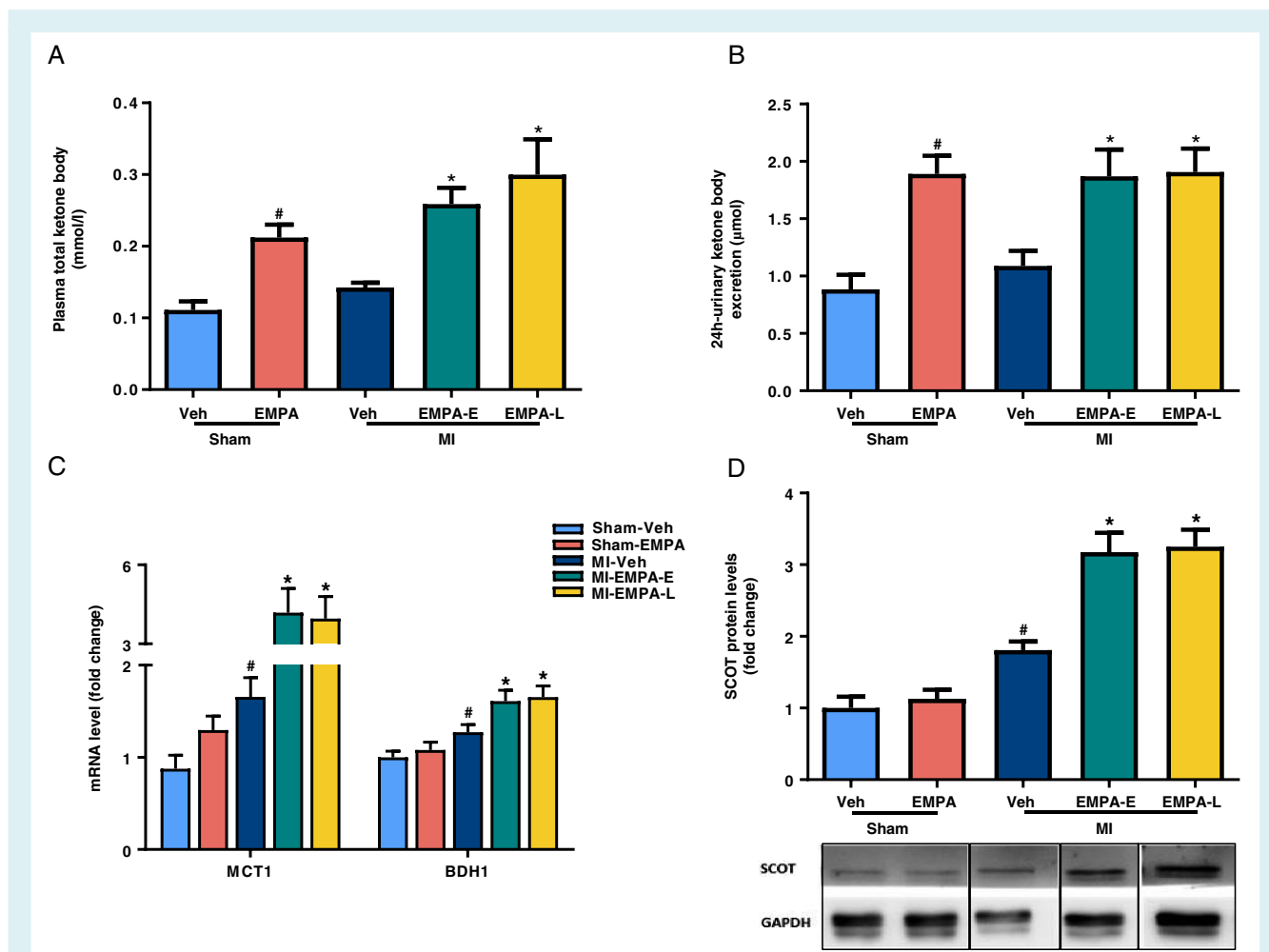


Figure 6 Effect of empagliflozin (EMPA) on ketone metabolism. (A) Plasma total ketone body levels. (B) 24 h urinary ketone body excretion. (C) Measurement of mRNA levels to assess ketone metabolism. (D) Protein levels of succinyl-CoA:3-ketoacid CoA transferase (SCOT). BDH1, ketogenic enzyme β -hydroxy butyrate dehydrogenase; EMPA-E, EMPA-early; EMPA-L, EMPA-late; MCT1, monocarboxylate transporter 1; MI, myocardial infarction; Veh, vehicle. Data are presented as means \pm standard errors of the mean. * $P < 0.05$ vs. MI-Veh; # $P < 0.05$ vs. Sham-Veh.

interesting hypothesis is that the natriuresis induced by EMPA attenuates sodium overload in cardiomyocytes through inhibition of the sarcolemmal sodium–hydrogen exchanger. Elevated intracellular sodium levels are thought to compromise mitochondrial calcium handling and thereby contribute to mitochondrial dysfunction in HF.⁴⁶ The metabolic effects of EMPA observed in our study could thus partially reflect reductions in intracellular sodium. Nevertheless, it is hard to comprehend that the profound effects of EMPA on cardiac remodelling and cardiac metabolism that we observed are solely explained by their diuretic effects, as we did not observe a difference in blood pressure or LV filling pressures (online supplementary Table S2).

Another possible explanation for the beneficial effects of SGLT2i could lie in their effects on systemic and cardiac metabolism, which have consistently been observed in models of diabetes and heart disease. First, EMPA improves glycaemic control in diabetic patients and the caloric loss associated with increased urinary glucose excretion results in weight loss.^{47,48} Weight loss

is thought to improve myocardial insulin sensitivity, and myocardial insulin resistance is common in severe HF, irrespective of the presence of T2DM.³³ Second, several studies have suggested that SGLT2i can restore cardiac mitochondrial dysfunction. Indeed, the SGLT2i dapagliflozin restored cardiac PGC1- α in pre-diabetic rats.⁴⁹ PGC1- α is a critical mediator of mitochondrial biogenesis and the reductions in PGC1- α are thought to contribute to mitochondrial dysfunction observed in HF. Mizuno *et al.*³⁰ recently demonstrated that EMPA normalizes the size and number of mitochondria in diabetic hearts after a MI. This is consistent with our observation that EMPA restored mtDNA damage, mtDNA/nDNA ratio and restored PGC1- α expression, indicating that the effects of SGLT2i on mitochondrial biogenesis can be translated to the non-diabetic failing heart. Third, the changes in PDH activity and the expression levels of CPT1, GLUT4 and PDK4 observed by us and by others,⁴⁹ suggest that the myocardial capacity to oxidize glucose and fatty acids is improved by EMPA. Verma *et al.*⁵⁰ recently validated this concept by comparing substrate utilization in

a working heart model using hearts from EMPA or vehicle-treated diabetic rats. In accordance with our observations, these authors also showed that EMPA treatment restored myocardial glucose and fatty acid oxidation to non-diabetic levels. Fourth, during disease progression, diabetic and failing hearts increasingly rely on ketone bodies as a fuels source,^{37,38,51} and SGLT2i increase circulating ketone levels.³⁶ The myocardial capacity to metabolize ketone bodies is increased in HF, reflected by an increased expression of ketogenic enzymes such as BDH1.^{37,38} Ketones are considered to be more energy efficient under conditions of metabolic stress as they require less oxygen per molecule of ATP generated.^{37,38} In addition, ketone oxidation does not inhibit the oxidation of other substrates through the Randel cycle,⁵⁰ and ketones appear to improve myocardial blood flow.⁵² A single dose of EMPA was recently shown to increase myocardial ATP, which was strongly correlated with the changes in circulating ketone levels.⁵³ Our findings that EMPA increases the bioavailability and the myocardial utilization of ketone bodies which also increases ATP production in non-diabetic rats with LV dysfunction after MI are consistent with these observations. Together these data strongly suggest that the restoration of cardiac ATP levels by EMPA is at least partially caused by increased ketone oxidation. Of note, the result suggests that SGLT2-mediated refuelling of the heart contributes to the beneficial effects of EMPA on cardiovascular outcomes. Our study thus clearly demonstrates that the salutary effects of EMPA on cardiac metabolism that have been described in diabetic models also occur in a non-diabetic context.

Study limitations

First, our study provides a detailed insight into the myocardial effects of SGLT2 inhibition but was not designed to establish cause and effect of the proposed mechanisms responsible for the cardio-metabolic effects. Second, while we describe multiple molecular effect of EMPA in the myocardium, we did not establish the mechanisms underlying these molecular changes. More focused mechanistic studies with for instance BDH1 or SCOT knockout mice would be required for this purpose.⁵⁴ Third, we also did not provide direct proof that ketone body oxidation is increased in EMPA-treated hearts nor did we quantify the contribution of ketone body oxidation to the increase in myocardial ATP levels. This would require detailed metabolomics analysis, which is beyond the scope of the current manuscript. In addition, the effects of EMPA on ketone body utilization appear to be dependent on the severity of cardiac dysfunction as myocardial ketone utilization is not affected in mild HF.^{50,55} Ketone bodies may also have vasodilatory effects on the myocardium, which could explain part of the protective effects beyond myocardial ketone utilization.⁵² Finally, while the post-MI LV dysfunction models allow robust and predictable changes in cardiac remodelling and HF development, the effects of EMPA may be different in a clinical context. Nevertheless, our study provides unique insights into the cardiac effects of EMPA in the non-diabetic failing heart and supports the exploration of EMPA as a bona fide HF drug in patients with or without diabetes.

Conclusion

Empagliflozin favourably affects cardiac function and remodelling in non-diabetic rats with LV dysfunction after MI, associated with substantial improvements in cardiac ATP production. Importantly, it did so without renal adverse effects. Our data suggest that EMPA might be of benefit in HF patients without diabetes.

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Methods S1. Supplementary methods.

Figure S1. (A) Daily food intake. (B) Daily water intake. (C) Body weight at termination.

Figure S2. (A) % Haematocrit levels. (B) Plasma glucose levels. (C) Plasma sodium levels. (D) Plasma potassium levels.

Table S1. Primer sequences used for quantitative real-time polymerase chain reaction.

Table S2. Results of left ventricular haemodynamics measurements.

Table S3. Cardiac echo measurements.

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