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Angiotensin converting enzyme and sodium glucose cotransporter inhibitors alleviate inflammatory effects of SARS-CoV-2 in cardiomyocytes

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Coronavirus disease 2019 (COVID-19) patients frequently have cardiac involvement [1]. This is partly attributed to the abundant expression of angiotensin-converting enzyme 2 (ACE2), functional receptor of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in cardiomyocytes [2]. There are concerns regarding angiotensin-converting enzyme inhibitor (ACEi) use amid the pandemic as ACEi is postulated to upregulate ACE2 expression and increase susceptibility to SARS-CoV-2 myocardial damage [3]. Likewise, the use of sodium-glucose transport protein 2 inhibitors (SGLT2i) in diabetic COVID-19 patients is controversial. Diabetic societies recommend withholding SGLT2i if hospitalized for COVID-19 to reduce risk of diabetic ketoacidosis. In a stark contrast, investigators have been exploring the use of SGLT2i in COVID-19 patients, such as the Dapagliflozin in Respiratory Failure in Patients with COVID-19 (DARE-19) trial, owing to its potent cardiovascular protective effects [4]. To date, there is a lack of experimental data to guide ACEi and SGLT2i use among COVID-19 patients.

Recently, the present team [5] and others recapitulated myocardial damage of SARS-CoV-2 in induced-pluripotent stem cell-derived cardiomyocytes (iPSC-CM). SARS-CoV-2 causes myocardial damage by exerting direct cytopathogenic effects and inducing inflammation via cytokines/chemokines expression [5]. In the present study, an investigation of the effects of ACEi and SGLT2i pre-treatment on myocardial ACE2 expression, susceptibility to SARS-CoV-2 infection and cardiomyocytes viability using an iPSC-CM platform.

The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB-UW08-258 and UW 16-365 20-07-2016). Written informed consent was obtained from the participant. The iPSCs used were derived from a healthy Chinese volunteer. Detailed methods of iPSC generation, characterization, and *in vitro* cardiomyocyte differentiation used were previously reported by us [5, 6]. Approximately 3×10^4 and 1×10^4 iPSC-CM were plated into 24-well and 96-well culture dishes pre-coated with Matrigel plate

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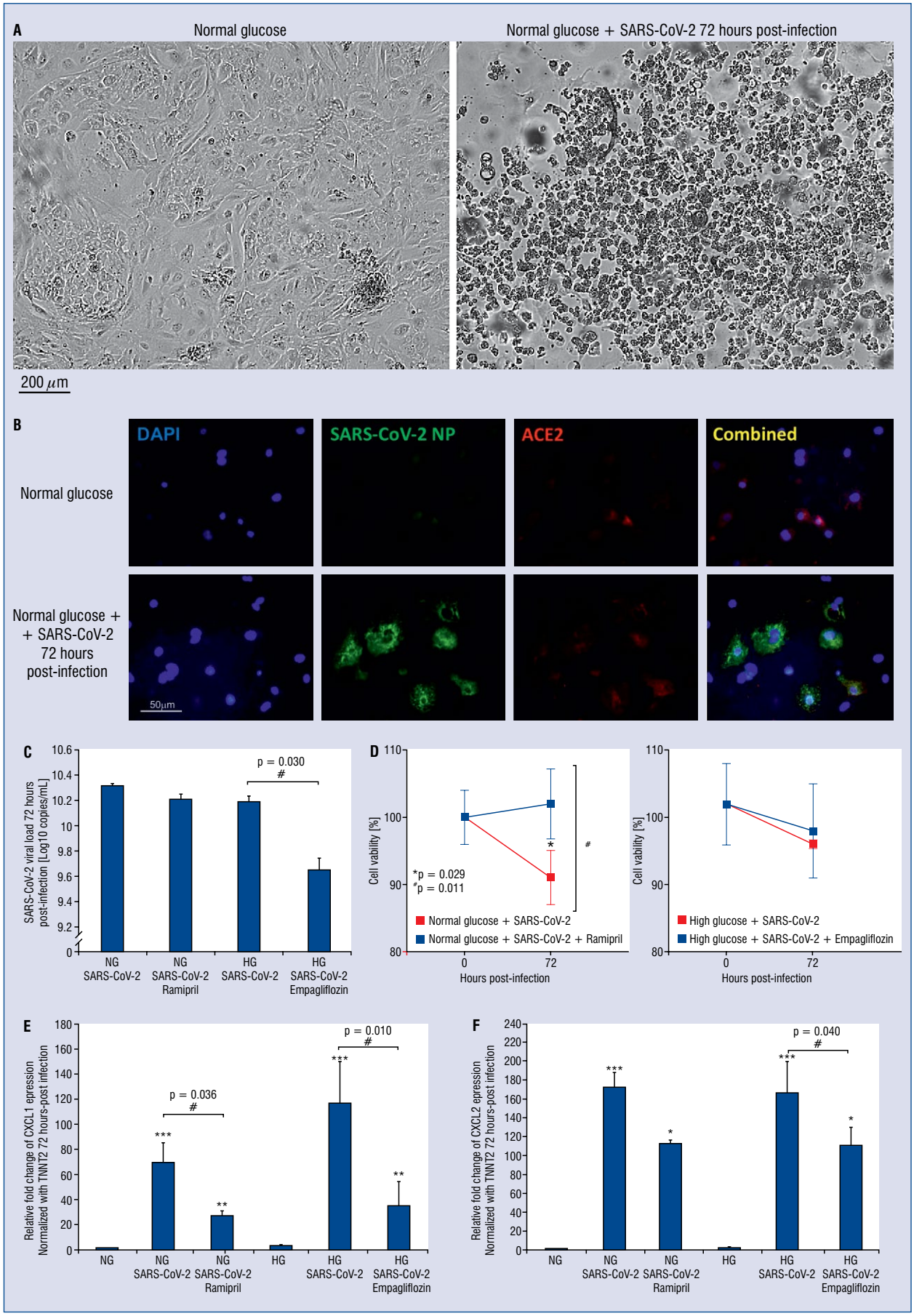
A
Normal glucose
Normal glucose + SARS-CoV-2 72 hours post-infection
200 μm
B
DAPI
SARS-CoV-2 NP
ACE2
Combined
Normal glucose
Normal glucose + + SARS-CoV-2 72 hours post-infection
50μm
C
SARS-CoV-2 viral load 72 hours post-infection [Log10 copies/mL]
p = 0.030
#
NG SARS-CoV-2
NG SARS-CoV-2 Ramipril
HG SARS-CoV-2
HG SARS-CoV-2 Empagliflozin
D
Cell vability [%]
*p = 0.029
#p = 0.011
Normal glucose + SARS-CoV-2
Normal glucose + SARS-CoV-2 + Ramipril
Hours post-infection
High glucose + SARS-CoV-2
High glucose + SARS-CoV-2 + Empagliflozin
E
Relative fold change of CXCL1 epression Normalized with TNNT2 72 hours-post infection
p = 0.036
p = 0.010
F
Relative fold change of CXCL2 epression Normalized with TNNT2 72 hours-post infection
p = 0.040

Figure 1. Effect of cardiometabolic drugs on induced-pluripotent stem cell-derived cardiomyocytes (iPSC-CM) infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); **A.** Brightfield microscopy with cytopathic changes of iPSC-CM including cell clumping and detachment from the culture dish at 72 hours post-infection (hpi); **B.** Immunofluorescence studies (blue: DAPI; green: SARS-CoV-2-NP, SARS-CoV-2 nucleocapsid protein; and red: angiotensin converting enzyme 2 [ACE2]) showing SARS-CoV-2 nucleocapsid proteins within iPSC-CM cytoplasm; **C.** Empagliflozin pre-treatment resulted in reduced supernatant viral load by 0.54 Log_{10} copies/mL at 72 hpi comparing to positive control ($^{\#}p = 0.030$). Data represent mean ± standard error of the mean from triplicate experiments. Comparisons between two groups were performed using the Student t test. Number sign ($^{\#}$) indicates statistical significance between infection group and infection group with cardiometabolic drug ($^{\#}p < 0.05$); HG — high glucose; NG — normal glucose; **D.** Cell viability reduced from 100 ± 4.05% to 91.0 ± 4.00% (*p = 0.029) at 72 hpi in positive control. Cell viability modestly improved by 12.1% ($^{\#}p = 0.011$) in the ramipril group. Data represent mean ± standard error of the mean from triplicate experiments. Comparisons between two groups were performed using the Student t test. Number sign ($^{\#}$) indicates statistical significance between infection group and infection group with cardiometabolic drug ($^{\#}p < 0.05$); **E.** Relative quantification of reverse transcription quantitative polymerase chain reaction products were assessed using the $2^{-\Delta\Delta Ct}$ method, using troponin T (TNNT2) as internal control, where $\Delta\Delta Ct = [(Ct_{target\ gene} - Ct_{TNNT2})$ Treatment group $- (Ct_{target\ gene} - Ct_{TNNT2})$ Control group]. SARS-CoV-2 infection upregulated C-X-C Motif Chemokine Ligand 1 (CXCL1) to 69.2-fold (***p < 0.001) and 117-fold (***p < 0.001) at 72 hpi in normal and high glucose conditions respectively. CXCL1 was downregulated to 27.0-fold ($^{\#}p = 0.036$) and 34.7-fold ($^{\#}p = 0.010$) by ramipril and empagliflozin, respectively. Data represent mean ± standard error of the mean from triplicate experiments. Comparisons between two groups were performed using the Student t test. Asterisk (*) indicates statistical significance between control group and infection or cardiometabolic drug group. Number sign ($^{\#}$) indicates statistical significance between infection group and infection group with cardiometabolic drug; ***p < 0.001; **p < 0.01; $^{\#}p < 0.05$; HG — high glucose; NG — normal glucose; **F.** SARS-CoV-2 infection upregulated C-X-C Motif Chemokine Ligand 2 (CXCL2) to 172-fold (***p < 0.001) and 166-fold (***p < 0.001) in normal and high glucose conditions, respectively. CXCL2 was downregulated to 110-fold ($^{\#}p = 0.040$) by empagliflozin. Data represent mean ± standard error of the mean from triplicate experiments. Comparisons between two groups were performed using the Student t test. Asterisk (*) indicates statistical significance between control group and infection or cardiometabolic drug group. Number sign ($^{\#}$) indicates statistical significance between the infection group and infection group with cardiometabolic drug; ***p < 0.001; *p < 0.05; $^{\#}p < 0.05$; HG — high glucose; NG — normal glucose.

(Thermo Scientific, USA). To evaluate the effects of cardiometabolic medications, iPSC-CM were pretreated for 7 days with ACEi ramipril (0.1 μM) (Cayman, USA), or SGLT2i empagliflozin (5 μM) (Selleckchem, USA). To recapitulate hyperglycemic state of diabetic patients, high glucose environment (22 mM glucose) was used for empagliflozin [6]. *In vitro* infection was performed by applying SARS-CoV-2 to iPSC-CM monolayers with multiplicity of infection of 0.1 and incubating at 37°C for 1 hour. Experiments involving live SARS-CoV-2 were performed in Biosafety Level-3 Facility.

Seventy-two hours post-infection (hpi), iPSC-CM ceased spontaneous beating and demonstrated cytopathogenic changes with cell clumping and detachment from culture dish (Fig. 1A, B). After ramipril pre-treatment, ACE2 mRNA expression assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in iPSC-CM was upregulated to 5.87-fold ($p < 0.001$). To assess supernatant viral load, RT-qPCR targeting SARS-CoV-2 using forward primer 5'-CGCATACAGTCTTRCAGGCT-3' and reverse primer 5'-GTGTGATGTTGAWATGACATGGTC-3' was performed (Fig. 1C). Despite an increased ACE2 expression after ramipril pre-treatment, iPSC-CM susceptibility to SARS-CoV-2 was not enhanced with no significant increase in SARS-CoV-2 RNA comparing to positive control. Cell viability was assessed using colorimetric-based Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, USA) (Fig. 1D). IPSC-CM viability was quantified using relative absorbance at 450 nm and the absorbance in normal glucose condition without infection or medication was taken to be 100%. Cell viability reduced from 100 ± 4.05% to 91.0 ± 4.00% ($p = 0.029$) at 72 hpi in positive control. Intriguingly, ramipril improved cell viability by 12.1% ($p = 0.011$) comparing to positive control albeit comparable viral load. Unlike ramipril, empagliflozin did not significantly affect ACE2 expression. Empagliflozin caused a modest reduction of supernatant SARS-CoV-2 viral load by 0.54 Log_{10} copies/mL ($p = 0.030$) and downregulated natriuretic peptide B (NPPB) mRNA expression from 3.21-fold to 1.96-fold ($p < 0.05$) comparing to positive control. Nonetheless, empagliflozin treatment did not affect iPSC-CM viability.

SARS-CoV-2 infection causes myocardial damage partly by upregulating expression of pro-inflammatory cytokine/chemokines, particularly

C-X-C Motif Chemokine Ligand 1 (CXCL1) and C-X-C Motif Chemokine Ligand 2 (CXCL2) [5]. Of note, the CXCL1-CXCR2 axis was known to mediate monocytic infiltration to the myocardium [7]. CXCL1 and CXCL2 mRNA expression in iPSC--CM increased to 69.2-fold ($p < 0.001$) and 172-fold ($p < 0.001$) in a normal glucose culture condition and 117-fold ($p < 0.001$) and 166-fold ($p < 0.001$) in a high glucose condition. Interestingly, ramipril attenuated SARS-CoV-2 induced CXCL1 expression to 27.0-fold ($p = 0.036$). Similarly, empaglifozin attenuated SARS-CoV-2 induced CXCL-1 and CXCL2 expression to 34.7-fold ($p = 0.010$) and 110-fold ($p = 0.040$) respectively (Fig. 1E, F).

In the present study, we exploited our recently established iPSC-CM platform to study the effects of ACEi and SGLT2i on ACE2 expression and SARS-CoV-2 susceptibility. We demonstrated that in concordance to previous animal models, ACEi treatment resulted in an upregulation of ACE2 expression in iPSC-CM. Counterintuitively, the ACEi-induced ACE2 upregulation in iPSC--CM did not lead to an increased susceptibility to SARS-CoV-2 infection. Plausibly, the abundance of ACE2 in iPSC-CM may have already been above the stoichiometry of entry for SARS-CoV-2 virus in baseline condition, thereby further increase in ACE2 expression with ramipril did not further increase SARS-CoV-2 cellular entry. In fact, ACEi treatment improved iPSC-CM survival upon SARS-CoV-2 infection and alleviated SARS-CoV-2 induced inflammatory response in iPSC-CM. This is in consistence with clinical observation that hospitalized patients taking ACEi appeared to have relative beneficial effects in terms of death or critical care unit admission [8]. One unexpected finding from our experiments was the potent anti-inflammatory effects of SGLT2i on SARS-CoV-2 infected myocardium. Pharmacological sodium-hydrogen exchanger isoform-1 (NHE-1) inhibition was shown to suppress nuclear factor kappa B (NF-κB) activity and proinflammatory response in endothelial cells stimulated by bacterial lipopolysaccharide [9]. As SGLT2i was shown to inhibit NHE-1 of cardiomyocytes [10], it is plausible that the marked downregulation of CXCL1 and CXCL2 were mediated through upstream suppression of NF-κB. Broadly speaking, the anti-inflammatory property of SGLT2i may also contribute to its potent effect against heart failure in diabetic patients, as diabetic cardiomyopathy is partly caused by myocardial inflammation. The current experiments had the following limitations: First, the experiments focused on the effects on the myocardium and its results cannot be directly extrapolated to other systems. Second, animal models will allow more holistic assessment of the systemic immune response.

Taken collectively, the results provided experimental evidence to support continuation of ACEi, and SGLT2i in stable diabetic patients amid the COVID-19 pandemic. The present findings also contributed to a better understanding of ACE2 physiology in human hearts and anti-inflammatory effects of SGLT2i.

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Conflict of interest: None declared

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