Embryonic Cardioprotection by Hydrogen Sulphide: Studies of Isolated Cardiac Function and Ischaemia-Reperfusion Injury in the Chicken Embryo *

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KEY POINTS

- In mammals, pregnancy complications can trigger an embryonic or fetal origin of cardiac dysfunction. However, underlying mechanisms remain uncertain because the partial contributions of the challenge on the mother, placenta or offspring are difficult to disentangle.

- The avian embryo permits isolation of the direct effects of suboptimal conditions during development on the cardiac function of the offspring, independent of additional effects on the mother and/or the placenta.

- Therefore, the objectives of this work were to adapt the isolated Langendorff technique using the chicken embryo to study the physiology of the developing heart.

- Here, we introduce the novel technology and show the utility of the technique exploring cardioprotective roles of H_2S in the chicken embryo heart. This work lays the foundation to study the direct effects of H_2S therapy on the embryonic heart independent of effects on the mother and the placenta in adverse development.

ABSTRACT

This study adapted the isolated Langendorff preparation to study the chicken embryo heart in response to ischaemia-reperfusion (IR) injury. The utility of the technique was tested by investigating cardioprotective effects of hydrogen sulphide (H_2S) and underlying mechanisms. Embryonic hearts (19 out of 21 days of incubation) mounted on a Langendorff preparation were exposed to IR (30 min ischaemia) after 4 treatments administered randomly, all as a 1mM bolus, into the perfusate: saline vehicle (control); sodium hydrogen sulphide (NaHS); NaHS plus glibenclamide, an antagonist of K_{ATP} opening (NaHS Glib), and Glib alone (Glib). Relative to controls, NaHS treatment improved cardiac function after ischaemia (mean±SD for under the curve, AUC, for left ventricular developed pressure, LVDP: 1767.3±929.5 *vs.* 492.7±308.1; myocardial contractility, dP/dT_{max}: 2748.9±1514.9 *vs.* 763.7 ±433.1) and decreased infarct size (22.7± 8.0 *vs.* 43.9±4.2%) and cardiac damage (% change in creatinine kinase, 49.3±41.3 *vs.* 214.6 ±155.1; all $P<0.05$). Beneficial effects of NaHS were blocked by Glib. Glib alone had no effects. NaHS increased CFR during baseline (mean±SD for AUC: 134.3± 91.6 *vs.* 92.2±35.8) and post I/R (1467± 529.5 *vs.* 748.0±222.1; both $P<0.05$). However, this effect was not prevented by Glib. Therefore, the chicken embryo heart is amenable for study via the Langendorff preparation under basal conditions and during IR. The data show that H_2S confers embryonic cardiac protection via opening of myocardial K_{ATP} channels and not via increasing CFR. H_2S may prove a useful therapeutic agent to protect the human fetal heart against IR injury, as may occur in complicated labour.

INTRODUCTION

Complications during mammalian pregnancy, such as gestational diabetes, maternal smoking, pregnancy at high altitude or preeclampsia can trigger a fetal origin of heart disease (Fowden *et al.* 2006). However, underlying mechanisms have proven difficult to isolate because suboptimal conditions during pregnancy often affect the mother, the placenta as well as the developing offspring. In this regard, oviparous species, like the chicken, offer many advantages. Embryonic development occurs in the absence of either a mother or a placenta, nutrition is fixed within the egg, there is no need to control for within-litter variation or for effects on lactation, and many milestones of cardiac development are similar to humans (Itani *et al.* 2018). Therefore, the chicken embryo is ideally placed to isolate direct effects of adverse developmental conditions and of therapy on an early origin of heart disease. Therefore, the objectives of this work were to adapt the isolated Langendorff technique using the chicken embryo to study the physiology of the developing heart under basal conditions during ischaemia-reperfusion (IR) injury. The validity of the technique was tested by investigating potential cardioprotective agents against IR and underlying physiological mechanisms.

Growing evidence suggests that H_2S is vital to cardiovascular health (Shen *et al.* 2015). For instance, clinical studies report that a decrease in endogenous H_2S levels is linked to age-related cardiovascular pathology (Jiang *et al.* 2005; Polhemus *et al.* 2014; Perridon *et al.* 2016). Moreover, work in animal models shows that supplementation with an exogenous H_2S donor protects the adult heart from ischaemic reperfusion (IR) injury (Johansen *et al.* 2006). However, whether H_2S confers protection in the developing heart before birth remains completely unknown.

Mechanisms underlying cardiac protection by H_2S may include an increase in coronary blood flow enhancing coronary reserve due to its vasodilator actions (Bhatia, 2005) and/or action on myocardial K_{ATP} channels (Shen *et al.* 2015). Under normal physiological conditions in the adult heart, K_{ATP} channels are closed (Lu *et al.* 2008). In response to a decrease in the ATP:ADP ratio, as experienced during an ischaemic challenge, myocardial K_{ATP} open. This preserves cardiac health via limiting calcium influx and energy expenditure (Lederer *et al.* 1989; Burke *et al.* 2008). Mutations in cardiac K_{ATP} channels have been identified in patients with dilated cardiomyopathy (Bienengraeber *et al.* 2004). Genetic and pharmacological disruption of K_{ATP} channels impairs recovery following IR and negates the beneficial effects of ischaemic preconditioning (Suzuki *et al.* 2001; Gumina *et al.* 2003; Kane *et al.* 2006). Conversely, K_{ATP} overexpression confers resistance to ischaemic injury (Du *et al.* 2006).

Episodes of IR can also present *in utero* for a range of reasons. These include periods of increased uteroplacental vascular resistance, such as during preeclampsia, or during compression of the umbilical cord in late gestation, as in complicated labour and delivery (Morrison, 2008; Giussani, 2016). A recent review by Bennet (2017) shows clearly that the preterm sheep fetus is remarkabky tolerant to episodes of ischaemia produced by complete occlusion of the umbilical cord, even those lasting for periods longer than 20 minutes. Despite the fetal heart being more resistant to ischaemia compared to the adult heart, insufficient oxygen supply to developing organs can have detrimental and long-term effects, particularly in metabolically active tissues, such as the fetal heart in late gestation (Li *et al.* 2003). Growing evidence derived from human clinical studies as well as from animal models links suboptimal oxygenation during fetal development with increased cardiac susceptibility in the neonatal period, as well as increased cardiac risk in the adult offspring (Paterson & Zhang, 2010; Giussani & Davidge, 2013). Therefore, it is clinically relevant to find possible therapeutic

targets and interventions to protect the developing heart against IR injury. Here, we tested the hypothesis that H_2S confers protection against IR injury in the embryonic heart via opening of K_{ATP} channels and not via increasing coronary flow. This work lays the foundation to study the direct effects of H_2S therapy on the embryonic heart independent of effects on the mother and the placenta in adverse development.

METHODS

Ethical Approval

All animal procedures conform with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. This research was approved under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB).

Data, Materials and Code Disclosure Statement

The data discussed herein can be obtained from the corresponding author upon request.

Animals

All experiments were performed in the same experimental season. Fertilized Bovans Brown eggs (Medeggs, Norfolk, UK) were weighed and incubated from day 1 under controlled normoxic conditions (21% O_2, 37.9°C, 45% humidity, 12:12 hr light:dark cycle, automatic rotation every hour, Masalles incubator Mod-75A with electronic servo-controlled humidity cool steam injection system HS-Auto-3.5L; Masalles, Barcelona, Spain). The levels of oxygen,

humidity, and temperature inside the incubators were continuously monitored (DD103 DrDAQ Oxygen Sensor, Pico Technology, St. Neots, UK).

Embryonic Langendorff Heart Preparation

On day 19 (term is 21 days) of incubation**,** chicks were humanely killed by cervical transection. The heart was rapidly excised and immediately placed in ice-cold Krebs–Henseleit Buffer (KHB, NaCl:120 mM, KCl:4.7 mM, $MgSO_2.7H_2O$:1.2 mM, KH_2PO_4:1.2 mM, $NaHCO_3$:25 mM, glucose:10 mM, $CaCl_2.2H_2O$:1.3 mM), then mounted onto the Langendorff apparatus which was maintained at constant physiological temperature (Figure 1). Great care needs to be taken when mounting the heart as the chicken embyo aorta is very fragile. Using an aortic cannula, which was made from a 19 G needle, this preparation achieves retrograde perfusion of the isolated heart (Niu *et al*. 2013). The perfusing solution was KHB (40°C, gassed with O_2:CO_2, 95:5%) delivered at a constant pressure of 40 cm H_2O and filtered through a 5 μm cellulose nitrate filter (Millipore, Bedford, MA, USA). A small incision in the left atrium was made to allow a small, flexible non-elastic balloon made from cling film to be inserted into the left ventricle. The balloon contained distilled water and was connected to a rigid water-filled catheter, and then to a calibrated pressure transducer (Argon Medical Devices, Plano, TX, USA). Therefore, this set up allowed continuous monitoring of cardiac function. To obtain a left ventricular end diastolic pressure (LVEDP) of 5-10 mmHg, a 100-μL Hamilton syringe was used to adjust the balloon volume to 30 μL (Giussani *et al*. 2012; Niu *et al*. 2013).

Basal Function

Isolated hearts were given 15 min to stabilise on the preparation, then baseline functional recordings were made using an IDEEQ data acquisition system (version 0-2.5.0, Maastricht, Netherlands). Measurements of cardiac function included heart rate (HR), left ventricular

developed pressure (LVDP), LV end diastolic pressure (LVEDP), the maximum first derivative (dP/dt_{max}) and minimum first derivative (dP/dt_{min}) of left ventricular pressure, the contractility index (dP/dt_{max} normalised to mean pressure at the point of dP/dt_{max}), and Tau (the isovolumetric relaxation time constant). In addition, coronary flow rate (CFR) was determined gravimetrically via measuring the volume of perfusate effluent over time (Niu *et al*. 2013).

Drug Administration

Hearts received treatment 10 min before ischaemia according to one of four randomly-allocated groups*:* Control (buffer), Control+ NaHS (Sodium hydrogen sulphide, a H_2S donor, Hosoki *et al.* 1997), Control + Glib (Glibenclamide, a non-selective inhibitor of K_{ATP} opening, Ripoll *et al.* 1993) or Control +NaHS+Glib. All drugs were given as a single bolus at 1mM concentration dissolved in 0.5 ml of buffer vehicle, 10 minutes prior to ischaemia. The dose of NaHS administration was adopted from our own pilot experiments which showed indices of cardiac protection of NaHS with this dose and regimen of administration. The rationale for using 1 mM Glib was that this would stoichiometrically balance with the NaHS dose. All drugs were obtained from Sigma-Aldrich, Dorset, UK. Cardiac function and CFR were measured following treatment, during and after IR.

Ischaemia/Reperfusion (IR) Protocol

10 minutes after treatment, half an hour of global ischaemia was induced by shutting off the supply of perfusate (Figure 1). Cardiac function was analysed at 10 min intervals during baseline and ischaemia, and at 0 min, 5 min, 15 min, 30 min and 60 min reperfusion time. Coronary flow rate was analysed at 10 min intervals during baseline and ischaemia, and at 0 min, 15 min, 30 min and 60 min reperfusion time. Perfusate samples were taken during baseline and at the end of the ischemic insult (at -30 and 0 min reperfusion time).

Infarct Size Analysis

Following 2h of reperfusion, the heart was taken off the preparation. After recording of the heart weight, the heart was cooled for 10 min at -20°C because this allowed the tissue to be sliced more easily in a semi-frozen state. The heart was then sliced transversely into 5 x 1.5 mm-thick sections using a custom made slicer (Figure 2A). Mid cardiac slices (Slice 3) were incubated with 0.1% triphenyltetrazolium chloride dissolved in a phosphate buffer [mixture of Na_2HPO_4 (77.4%) and NaH_2PO_4 (22.6%)] for 20 min at 37 °C. Mid cardiac slices were then scanned (Scanjet 300, Hewlett Packard, Cambridge, UK) and the % infarct size from this section was determined using Image J software (version 1.52, National Institute of Health, Bethesda, MD, USA; Figure 2B). To quantify the infarct size, the colour threshold function in ImageJ was used to filter the background and select the infarcted area (Figure 2B-F). ImageJ then reported the number of pixels in the infarcted area and the total slice area. Infacrt size was calculated as a percentage by dividing the number of pixels representing the infarcted area by the number of pixels representing the total slice area.

Perfusate analysis

Coronary effluent samples (2 mL) were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Perfusate creatine kinase (CK) activity was analysed using a bichromatic coupled enzyme reaction assay (Siemens Dimension RxL analyser, Malvern, PA), while lactate dehydrogenase (LDH) activity was assessed using a colorimetric assay (Siemens Dimension RxL analyser, Malvern, PA). Activity of these enzymes are established markers of cardiac injury (Niu *et al*. 2018) and were expressed as % change from baseline.

Statistical analysis

Appropriate power calculations derived from previous data sets were performed to determine the minimum sample size required to achieve statistical significance set at $P<0.05$ (Mulder *et al.* 2002). Scientists measuring outcomes were blinded to treatments. All data are expressed as mean±SD. Comparisons were assessed for statistical significance using two- or three-way ANOVA. If interactions between main factors were significant, the Tukey *pot hoc* test was used to isolate differences. For all comparisons, statistical significance was taken when $P<0.05$.

RESULTS

Biometry and Basal Cardiac Function

Body weight, heart weight and relative heart weight of chicken embryos showed no significant difference between experimental groups (Table 1). Baseline cardiac function did not differ significantly between experimental groups (Table 1). Administration of the drugs or vehicle had no significant impact on heart rate, or indices of systolic and diastolic function prior to ischaemia, with the exception of coronary flow rate (Table 2). Coronary flow rate increased after NaHS with or without Glib during baseline conditions prior to ischaemia (Table 2, NaHS effects: $p<0.0001$; Glib effects: $p=0.6162$, with no interaction between main effects: $p = 0.1812$).

Cardiac Function Following Ischaemia

In control embryos, ischaemia led to significant depression of chronotopic (HR) and inotropic (LVDP) function (Figure 3A-D), as well as cardiac contractility (dP/dt_{max}) and relaxability (dP/dt_{min}) during reperfusion (Figure 4A-D). Administration of NaHS prior to ischaemia restored cardiac function towards basal levels post IR (Figure 3A-D and 4A-D), with the AUC of cardiac recovery significantly greater for all four cardiac functional variables in Con+NaHS vs. controls (Figures 3B and 3D, 4B and 4D). When NaHS was co-administered with Glib, there was no longer any cardioprotective effects (Figures 3A-D and 4A-D). Treatment with Glib alone did not have any significant effect on cardiac functional recovery (Figure 3A-D and 4A-D).

Coronary Flow Rate

CFR normalised to chicken embryo heart weight (Table 1 and Figure 5) did not differ between experimental groups. There was an increase in relative CFR following NaHS treatment during baseline, prior to ischaemia (Figure 5A). This increase in relative CFR following NaHS treatment was maintained post-ischaemia; values for AUC recovery in relative CFR were significantly greater than controls (Figures 5A and 5B). Here, the effects of NaHS on relative CFR were not altered when co-administered with Glib (Figures 5A and 5B). Treatment with Glib alone had no effect on relative CFR (Figures 5A and 5B).

Infarct Size

The IR protocol yielded a myocardial infarct size of 43.9±4.2 % in control embryo hearts, which was significantly reduced to 22.7±8.0 % with NaHS treatment prior to ischaemia (Figure 6A). This beneficial effect of NaHS was blocked when NaHS was given with Glib (Figure 6A). Treatment with Glib itself had no significant impact on infarct size (Figure 6A).

Perfusate Analysis

Relative to controls, values for CK and LDH were significantly lower post-ischaemia in the NaHS treatment group (Figure 6B and 6C). These benefits of NaHS on ischaemic cardiac injury were no longer occured when Glib was co-administered (Figures 6B and 6C). Treatment with Glib alone showed a relative change in CK and LDH equivalent to controls (Figure 6B and 6C).

DISCUSSION

In this study, we adapted the isolated Langendorff preparation to study the physiology of cardiac function during basal conditions and during IR injury using the chicken embryo heart in late incubation. This technology will be useful to isolate the direct effects of adverse conditions during development on embryonic cardiac function independent of effects on the maternal and/or placental physiology, highlighting the conceptual advance to the field of early origins of heart disease and developmental programming. We also provide novel evidence to support the hypothesis that H_2S has cardioprotective effects in the embryonic heart against IR

injury and that the mechanism of protection involves opening of myocardial K_{ATP} channels, independent of vsodilator effects of H_2S on coronary flow.

Infarct size is an established global indication of cardiac cell viability (Zhao & Vinten-Johansen, 2002). Analysis of infarct size at the end of our protocol shows that treatment with a H_2S donor (NaHS) prior to IR almost halved the extent of infarct size. Therefore, H_2S appears to significantly protect the developing heart from infarction. Moreover, data in the present study suggest that H_2S-mediated infarct protection in the embryonic heart relies on the opening of K_{ATP} channels, given that the NaHS-mediated reduction in infarct size is negated when co-administered with Glib, an antagonist of K_{ATP} opening. Perfusate analysis in this study considered the change in LDH and CK pre- and post-ischaemia. LDH is involved in glucose metabolism, catalysing the inter-conversion of lactate and pyruvate. In turn, CK catalyses the conversion of creatine to phosphocreatine, responsible for ATP transfer and utilization (Danese & Montagnana, 2016). When cardiomyocytes are damaged, LDH and CK are released from the cytoplasm into the bloodstream and utilised as clinical biomarkers for cardiac injury (Danese & Montagnana, 2016). In our study, the increase in perfusate concentration in LDH and CK post-ischaemia in control hearts provides strong biochemical evidence for embryonic cardiac damage resulting from IR injury. Moreover, our data suggest H_2S can protect against this cardiac injury, with values for LDH and CK significantly lower when NaHS was given prior to ischaemia. This H_2S-mediated cardioprotection appears to rely on opening of K_{ATP} channels, since treatment with both NaHS and the K_{ATP} antagonist Glib yielded a level of cardiac injury equivalent to controls.

In addition to these two biochemical measures of cardiac health, our study considered the functional implications of an IR challenge on the developing heart. All variables investigated,

including chronotropic (HR) and inotropic (LVDP) function as well as left ventricular contractility (dP/dt_{max}) and relaxability (dP/dt_{min}) were impaired by the IR protocol in control hearts. By contrast, pre-treatment with NaHS improved the recovery of all variables measured, indicating that the cardioprotective effects of H_2S translate onto a functional level. Our study also suggests that H_2S-mediated protection of embryonic cardiac function depends on the opening of K_{ATP} channels, as co-administration of NaHS with Glib prior to ischaemia dimnished the functional protection conferred by NaHS.

It is established that H_2S has vasodilator properties (Bhatia, 2005), an effect confirmed in our experiment. The data in the present study showed that CFR, independent of the heart size, significantly increased during baseline immediately following treatment with NaHS. Further, this effect pwersisted after IR. Increased cardiac perfusion maximises the supply of oxygen and nutrients to the myocardium. Therefore, it could be argued that the cardioprotective effects of H_2S are due to its vasodilator actions rather than other mechanisms. However, co-administration of NaHS with Glib did not affect the coronary dilator effects while it prevented the cardioprotective effects. Therefore, these data strongly support that the cardioprotective effects of H_2S are due to opening of myocardial K_{ATP} channels, independent of effects of NaHS on coronary flow. The data also suggest that the mechanism underlying the dilator effects of NaHS on CFR does not involve myocardial K_{ATP} channels.

Glibenclamide (Glib) is a member of the sulfonylurea family, which blocks K_{ATP} channels (Luzi & Poizza, 1997; Negroni *et al*. 2007). In the pacncreatic β-cell membrane, Glib reduces the conductance of the K_{ATP} channel and the reduced K^+ efflux determines membrane depolarization and influx of Ca^{2+} that promotes insulin secretion (Luzi & Poizza, 1997; Negroni *et al*. 2007). Consequently, Glib has been used for many years in the treatment of

Type II diabetes (Luzi & Poizza, 1997; Negroni *et al*. 2007). In the adult heart, the actions of Glib have mixes reviews. On one hand, blockade of myocardial K_{ATP} channels enhances Ca^{2+} influx and prolongs action potential duration and myocardial contractility, which has been deemed harmful, particularly during episodes of ischaemia (Khatib & Boyett, 2003; Negroni *et al*. 2007). On the other, studies have decsribed Glib as anti-arrythmogenic with antifibrillatory effects, associating these cardiac beneficial effects to a reduction in the efflux of K^+ induced by ischaemia (Luzi & Poizza, 1997). In the present study, administration of Glib alone prior to IR had no significant effect on all biochemical and functional assays of cardiac health when compared to controls in the chicken embryo. Our interpretation is that since cardiac K_{ATP} channels are closed at rest and only open when ATP levels fall, such as during ischaemia (Lu *et al.* 2008), administration of a drug that inhibits K_{ATP} channel opening when they are already closed prior to ischaemia should have negligible effects. Alternatively, lack of any effects of Glib on cardiac function in the present study may be because of differences between the embryonic and adult heart, or due to the mode of administration used. While a single bolus dose of Glib was sufficient to offset the actions of NaHS in the chicken embryo heart when administered together, when Glib was given alone, a bolus dose may have been too short-lived compared to an infusion to induce any noticeable effects on cardiac function before or during IR.

In the present study, it is interesting that the protective effects of NaHS on indices of systolic and diastolic cardiac function in the chicken embryo heart were most prominent in the first 30 min post-ischaemia and that this protection on cardiac function disappeared by 60 min of reperfusion. Despite this, pre-treatment with NaHS had a significant impact on infarct size, reducing it by half, when measured two hours after the end of the ischaemic insult. Combined, therefore, these data suggest that protection of cardiac function during the early period of

reperfusion is important in reducing infarct size. The limited capacity of NaHS in conferring protection on cardiac function longer-term may be due to the mode of its administration as a bolus pre-ischaemia rather than a constant infusion into the perfusate. Most drug regimes in clinical practice involve single/multiple dose administration rather than a continuous infusion. While the results of the bolus mode of administration provides proof-of-concept and illustrates preconditioning against IR, continuous infusion with more conservative doses may confer longer-term protection on cardiac function as well as infarction, with important implications for human clinical translation.

Episodes of asphyxia or ischaemia occur in embryonic or fetal life and they remain an unfortunately common clinical concern (Vannucci & Perlman, 1997; Giussani, 2016; Bennet, 2017; Ellery *et al.* 2018). Moreover, compromised blood supply to the fetal heart has been shown to disrupt normal cardiac development and programme an increased risk of hypertension in the adult offspring (Patterson & Zhang, 2010; Giussani & Davidge, 2013). Thus, there is clear clinical relevance to identify interventions that may prevent developmental cardiac ischaemic injury and an early origin of cardiac dysfunction. In this study, we have adapted the isolated Langendorff technique to study the chicken embryo heart in late incubation. The *ex vivo* chicken embryo heart model is not only amenable to functional study, but also to biochemical analysis of cardiac damage and infarct size. Our discoveries reveal that supplementation with the endogenous gasotransmitter H_2S donor NaHS confers significant direct protection to the embryonic heart during an IR challenge, independent of the presence of a mother or a placenta. Further, we have identified the opening of cardiac K_{ATP} channels independent of changes in coronary flow being central to the mechanism underlying H_2S-mediated direct cardioprotection. Therefore, supplementation with H_2S donors offers potential therapy to protect the developing heart directly against cardiac damage as a result of IR injury

in offspring of complicated pregnancy, independent of additional possible effects on the maternal and/ort placental physiology. We acknoweldge that techniques, such as echocardiography and cardiac MRI can offer advantages of studying the embryonic or fetal heart *in vivo*. Further, there is increasing interest in how adverse conditions during fetal development may affect fetal cardiac function in mammalian pregnancy in relation to gestational timing, duration and severity (Darby *et al*. 2020). Therefore, future studies ought to investigate different modes and doses of administration of candidate cardioprotective therapeutic agents, such as H_2S donors, during the embryonic or fetal periods not only during healthy conditions but also in development complicated by suboptimal conditions, such as with chronic hypoxia, excess glucocorticoid exposure or infection. Sudies should ideally compare *ex vivo* with *in vivo* techniques to assess embryonic or fetal cardiac function, using different species with the longterm goal of translating rational therapies to the human clinical condition.

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AUTHOR CONTRIBUTIONS

The experiments were performed at The Barcroft Centre at The University of Cambridge. YN, RH and DG were involved in the conception and design of the work, data acquisition, analysis and interpretation of data for the work, drafting the work and revising it critically for important intellectual content. TG, KB and SF were involved in the the conception and design of the work, data analysis and its interpretation.

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FIGURES LEGENDS

Figure 1. Summary of the experimental protocol. Hearts were isolated from 19-day old chicken embryos and perfused using a Langendorff preparation. Hearts were perfused with Krebs-Henseleit bicarbonate buffered solution in a retrograde fashion via the aorta at the constant pressure of 40 cmH_2O, gassed with O_2: CO_2 (95% : 5%). A small flexible non-elastic balloon made from cling film was inserted into left ventricle via the left atrium. The balloon contained distilled water and was connected to a rigid water-filled catheter and a calibrated pressure transducer. Indices of left ventricular function were continuously recorded throughout the protocol. After basal recording, a 30 minute global ischaemic challenge was introduced followed by 120 minutes of reperfusion. Sodium hydrosulfide (NaHS) and / or glibenclamide (Glib) were administered as a bolus at a dose of 1mM 10 minutes prior to onset of ischaemia. Perfusate was collected before and after ischaemia to determine concentrations of LDH (lactate dehydrogenase) and CK (creatine kinase), two established markers of cardiac injury. At the end of the experiment, the heart was sliced and slices were stained with triphenyltetrazolium chloride for the measurement of infarct size.

Figure 2. Heart slicer and measurement of infarct size. A, shows a custom made slicer for use with the chicken embryo heart; B, shows an example of a mid cardiac section showing white infarcted tissue, in contrast to healthy tissue which appears red; C, shows how one can filter all healthy tissue using the colour-threshold function in ImageJ; D, shows how to select an area of interest; E, shows how the parameters of the colour-threshold function in ImageJ could be adjusted so that only infarcted tissue is filtered; F, shows how the area of the infarct area can then be selected for measurement.

Figure 3. NaHS treatment improves cardiac chronotropic and inotropic recovery after an ischaemic challenge via opening KATP channels. Values are mean ± SD. A, heart rate (HR) recovery expressed as a percentage of the pre-ischaemic level; B, area under the curve of HR recovery; C, left ventricular developed pressure (LVDP) recovery expressed as a percentage of the pre-ischaemic level, and D, area under the curve of LVDP recovery. Groups are control (Con, white, n=8), Con with NaHS treatment (Con+NaHS, green, n=8), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, red, n=8), and Con with Glib treatment (Con+Glib, blue, n=8). NaHS and / or glibenclamide were administered as a bolus at a dose of 1mM 10 minutes prior to onset of ischaemia (arrow). Comparisons were assessed for statistical significance using three-way ANOVA (A and C) or two-way ANOVA (B and D). If interactions between main factors were significant, the Tukey *pot hoc* test was used to isolate differences: *P<0.05, Con *vs.* Con+NaHS; †P<0.05, Con+NaHS *vs.* Con+NaHS+Glib.

Figure 4. NaHS treatment improves cardiac contractility and relaxability after an ischaemic challenge via opening KATP channels. Values are mean ± SD. A, The maximum first derivatives of the LV pressure (dP/dt_{max}) recovery expressed as a percentage of the pre-ischaemic level; B, area under the curve of dP/dt_{max} recovery; C, The minimum first derivatives of the LV pressure (dP/dt_{min}) recovery expressed as a percentage of the pre-ischaemic level, and D, area under the curve of dP/dt_{min} recovery. Groups are control (Con, white, n=8), Con with NaHS treatment (Con+NaHS, green, n=8), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, red, n=8), and Con with Glib treatment (Con+Glib, blue, n=8). NaHS and / or glibenclamide were administered as a bolus at a dose of 1mM 10 minutes prior to onset of ischaemia (arrow). Comparisons were assessed for statistical significance using three-way ANOVA (A and C) or two-way ANOVA (B and D). If interactions between

main factors were significant, the Tukey *pot hoc* test was used to isolate differences: *P<0.05, Con *vs.* Con+NaHS.

Figure 5. NaHS treatment increases coronary flow rate independent of opening of KATP channels. Values are mean ± SD. A, Coronary flow rate (CFR) relative to heart weight (HW); B, area under the curve of the relative coronary flow rate. Groups are control (Con, white, n=7), Con with NaHS treatment (Con+NaHS, green, n=7), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, red, n=7), and Con with Glib treatment (Con+Glib, blue, n=7). NaHS and / or glibenclamide were administered as a bolus at a dose of 1mM 10 minutes prior to onset of ischaemia (arrow). Comparisons were assessed for statistical significance using three-way ANOVA (A) or two-way ANOVA (B). There were no significant interactions between main effects.

Figure 6. NaHS treatment reduces cardiac injury via opening KATP channels. Values are mean ± SD. A, cardiac infarct size expressed as a percentage of total area. Representative images and measurements are also shown; B, relative change from baseline in the concentration of creatine kinase (CK) at 0 minutes of reperfusion; C, relative change from baseline in the concentration of lactate dehydrogenase (LDH) at 0 minutes of reperfusion. Groups are control (Con, n=7-8), Con with NaHS treatment (Con+NaHS, n=8), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, n=7-8), and Con with Glib treatment (Con+Glib, n=7). NaHS and / or glibenclamide were administered as a bolus at a dose of 1mM 10 minutes prior to onset of ischaemia. Comparisons were assessed for statistical significance using two-way ANOVA. If interactions between main factors were significant, the Tukey *pot hoc* test was used to isolate differences: *P<0.05, Con *vs.* Con+NaHS; †P<0.05, Con+NaHS *vs.* Con+NaHS+Glib.

Table 1. Biometry and basal cardiac function

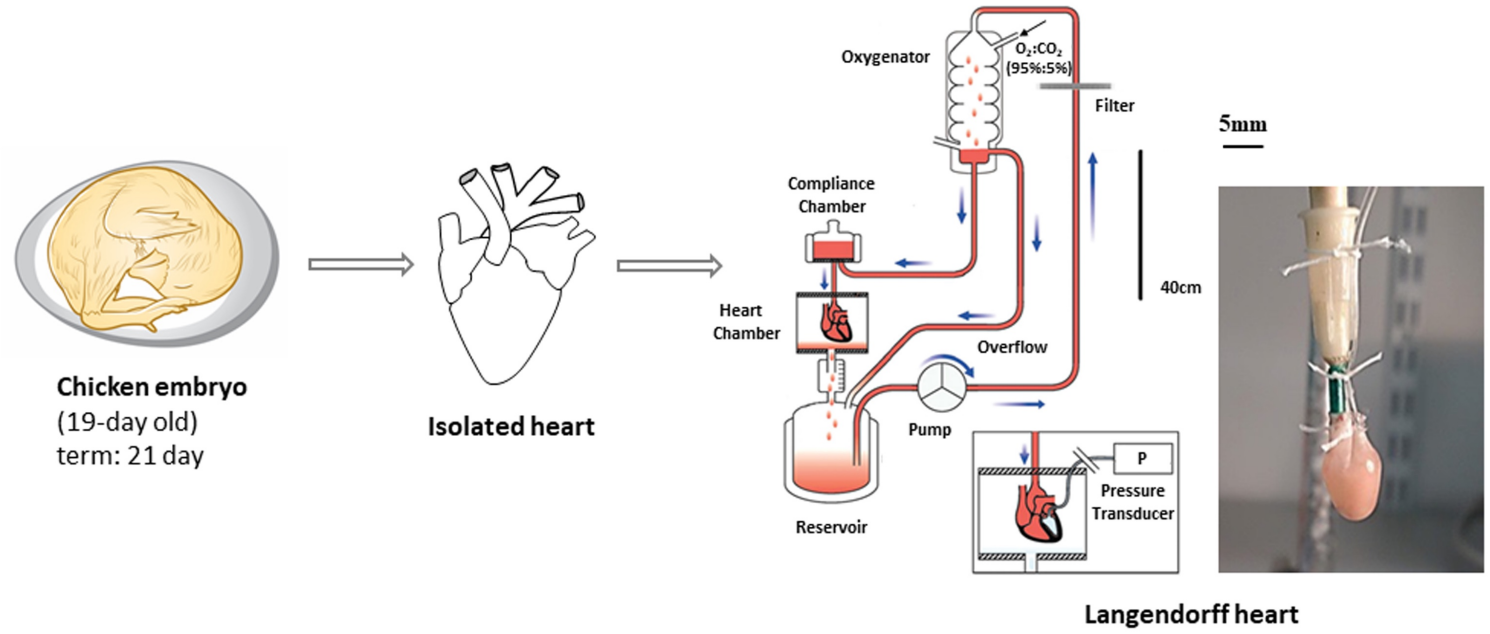
	Con	Con+NaHS	Con+NaHS+Glib	Con+Glib
Body weight (g)	20.21±3.31	20.37±3.27	19.17±2.31	20.05±1.50
Heart weight (g)	0.18±0.03	0.19±0.06	0.16±0.03	0.17±0.06
Relative heart weight (%)	0.89±0.14	0.89±0.24	0.86±0.28	0.86±0.25
HR (beats/min)	172.8±35.6	169.5±18.3	191.8±35.4	188.2±24.9
LVEDP (mmHg)	4.05±0.85	4.57±2.46	6.77±3.00	3.71±2.38
LVDP (mmHg)	21.23±3.82	20.21±6.15	17.68±3.51	20.68±6.12
dP/dt_{max} (mmHg/s)	405.7±106.1	428.9±105.3	381.8±49.6	424.7±129.5
dP/dt_{min} (mmHg/s)	468.5±116.8	469.0±108.6	432.8±69.9	487.4±142.0
CI (1/s)	34.29±6.51	36.56±15.12	34.03±17.23	40.53±9.70
Tau (s)	0.05±0.01	0.06±0.02	0.05±0.02	0.03±0.01
CFR/HW (ml/min/g)	6.41±1.27	6.17±1.80	6.56±1.49	6.23±2.60

Values are mean±SD. Groups are control (Con, n=8), Con with NaHS treatment (Con+NaHS, n=9), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, n=10), and Con with Glib treatment (Con+Glib, n=8). NaHS and / or glibenclamide were given as a bolus at dose of 1mM. LVEDP, left ventricular end diastolic pressure; LVDP, left ventricular developed pressure; dP/dt_{max}, the maximum first derivative of left ventricular pressure; dP/dtmin, the minimum first derivative of left ventricular pressure; CI, left ventricular contractility index; Tau, left ventricular relaxation time constant; CFR, coronary flow rate; HW, heart weight. There were no significant differences between groups.

Table 2. Cardiac function post drug treatment prior to ischaemia

	Con	Con+NaHS	Con+NaHS+Glib	Con+Glib
HR (beats/min)	175.9±26.3	161.9±19.8	190.5±37.3	181.4±22.6
LVEDP (mmHg)	3.90±1.33	4.13±3.69	4.29±3.42	2.18±1.78
LVDP (mmHg)	19.40±4.78	20.11±9.75	17.18±4.43	18.11±2.88
dP/dt_{max} (mmHg/s)	382.7±82.9	439.3±165.3	343.5±61.0	373.8±89.7
dP/dt_{min} (mmHg/s)	437.9±82.9	456.4±174.0	372.8±96.4	435.9±89.7
CI (1/s)	39.6±6.45	37.86±17.19	35.82±12.08	39.43±9.33
Tau (s)	0.04±0.01	0.07±0.03	0.06±0.03	0.04±0.02
CFR/HW (ml/min/g)	6.01±1.56	10.35±4.68	12.29±2.72	5.17±2.09

Values are mean±SD. Groups are control (Con, n=8), Con with NaHS treatment (Con+NaHS, n=9), Con with combined treatment of NaHS and glibenclamide (Con+NaHS+Glib, n=10), and Con with Glib treatment (Con+Glib, n=8). NaHS and / or glibenclamide were given as a bolus at dose of 1mM. LVEDP, left ventricular end diastolic pressure; LVDP, left ventricular developed pressure; dP/dt_{max}, the maximum first derivative of left ventricular pressure; dP/dtmin, the minimum first derivative of left ventricular pressure; CI, left ventricular contractility index; Tau, left ventricular relaxation time constant; CFR, coronary flow rate; HW, heart weight. Comparisons were assessed for statistical significance using two-way ANOVA. There were only differences in CFR/HW: NaHS effects: $p<0.0001$; Glib effects: $p=0.6162$, with no interaction between main effects: $p = 0.1812$.


Chicken embryo
(19-day old)
term: 21 day
Isolated heart
Oxygenator
O_2:CO_2
(95%:5%)
Filter
Compliance
Chamber
Heart
Chamber
Overflow
Pump
Reservoir
P
Pressure
Transducer
40cm
5mm
Langendorff heart

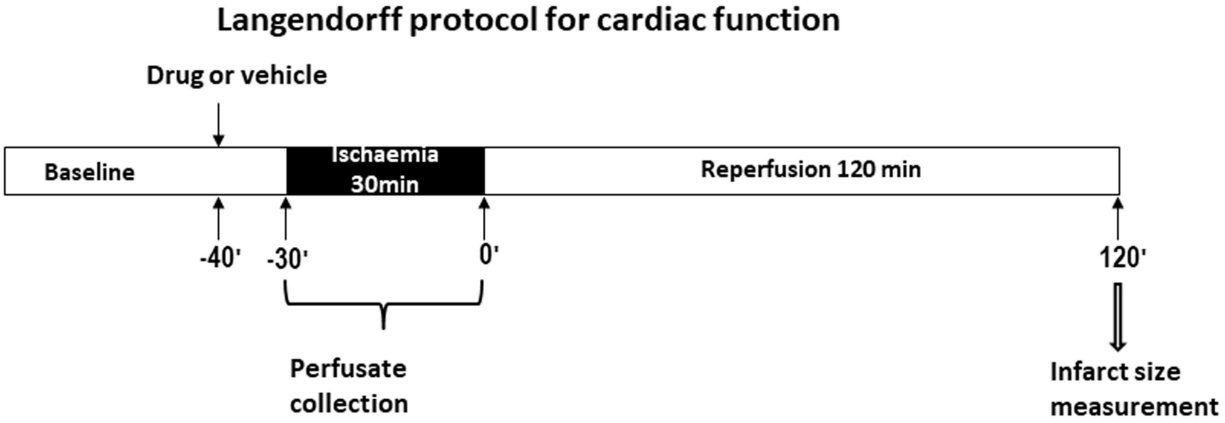

Langendorff protocol for cardiac function
Drug or vehicle
Baseline
Ischaemia
30min
Reperfusion 120 min
-40'
-30'
0'
120'
Perfusate
collection
Infarct size
measurement

Figure 1

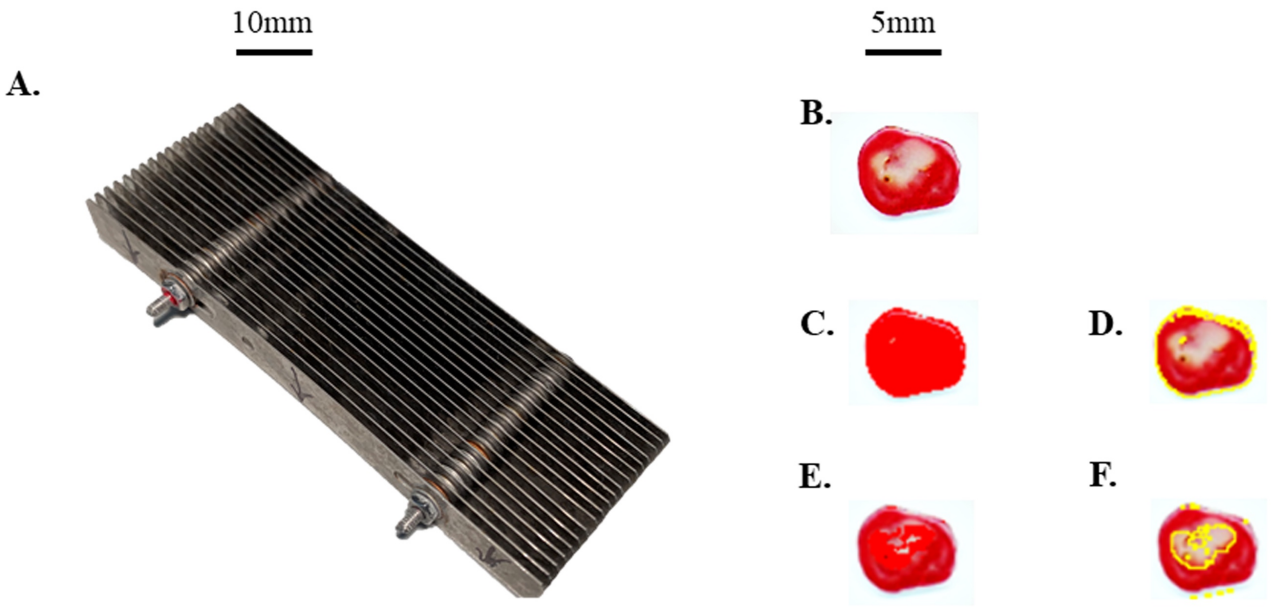


Figure 2

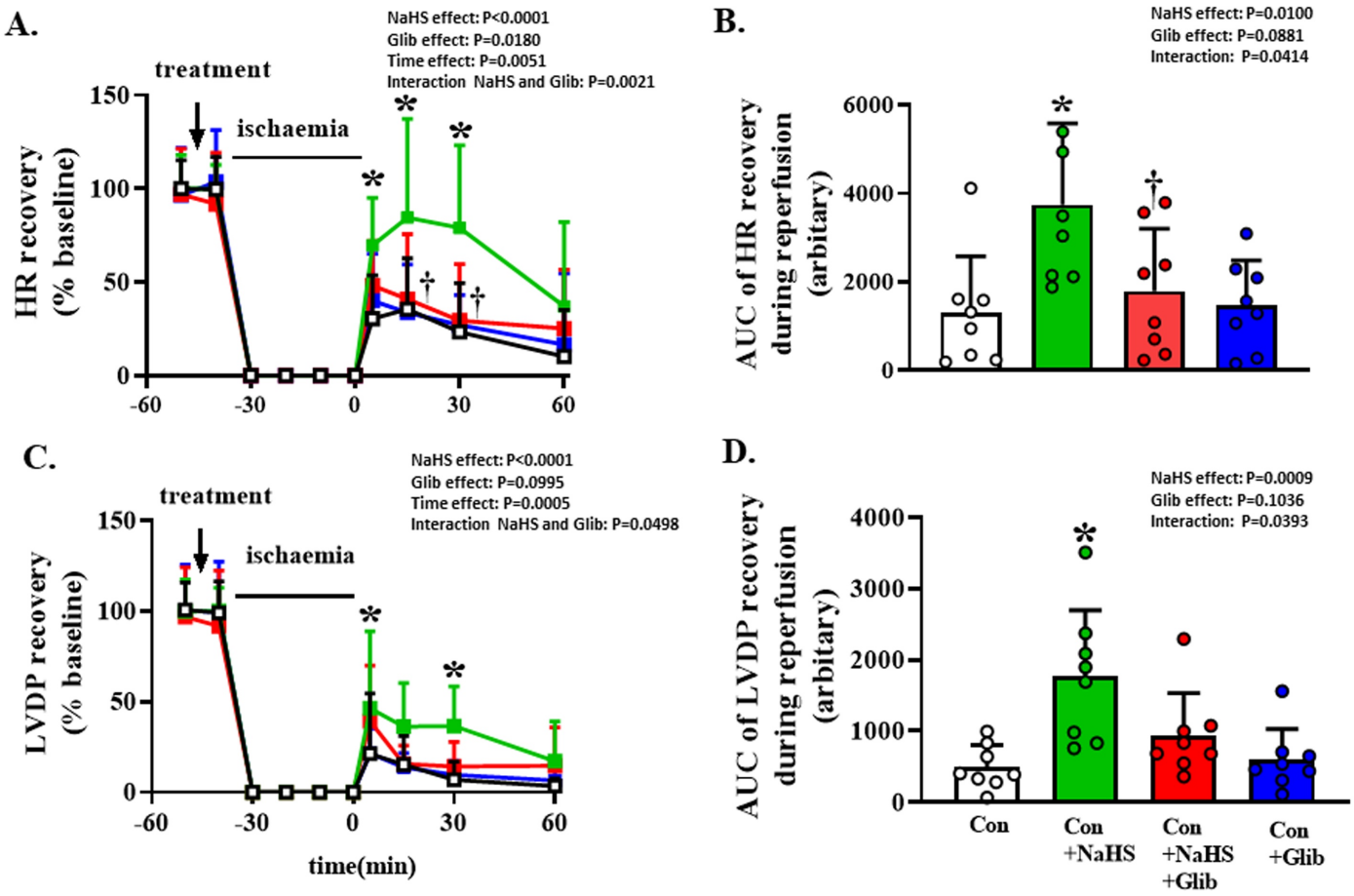

A.
NaHS effect: P<0.0001
Glib effect: P=0.0180
Time effect: P=0.0051
Interaction NaHS and Glib: P=0.0021
treatment
ischaemia
HR recovery (% baseline)
150
100
50
0
-60
-30
0
30
60
B.
NaHS effect: P=0.0100
Glib effect: P=0.0881
Interaction: P=0.0414
AUC of HR recovery during reperfusion (arbitary)
6000
4000
2000
0
C.
NaHS effect: P<0.0001
Glib effect: P=0.0995
Time effect: P=0.0005
Interaction NaHS and Glib: P=0.0498
treatment
ischaemia
LVDP recovery (% baseline)
time(min)
D.
NaHS effect: P=0.0009
Glib effect: P=0.1036
Interaction: P=0.0393
AUC of LVDP recovery during reperfusion (arbitary)
4000
3000
2000
1000
0
Con
Con +NaHS
Con +NaHS +Glib
Con +Glib

Figure 3

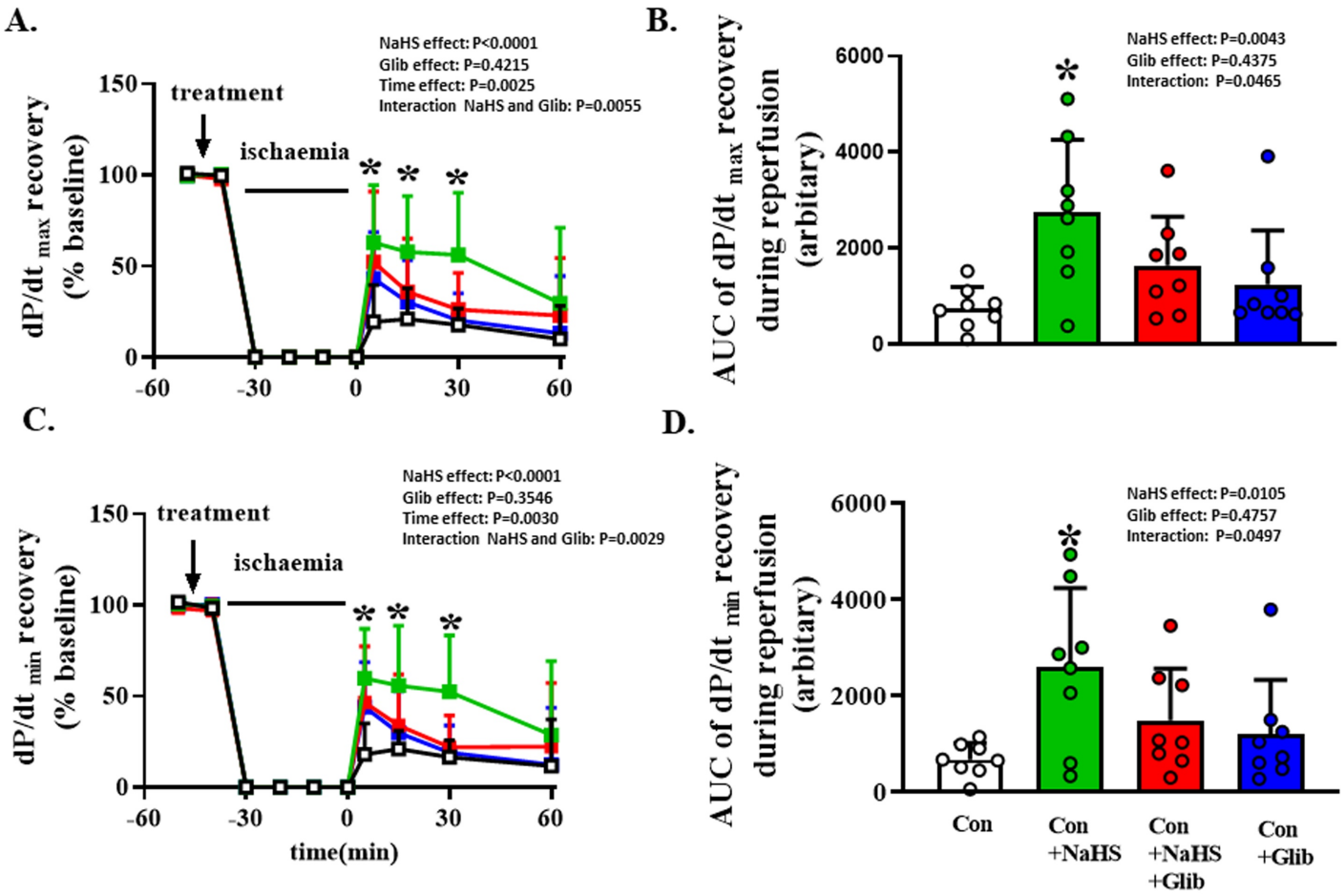
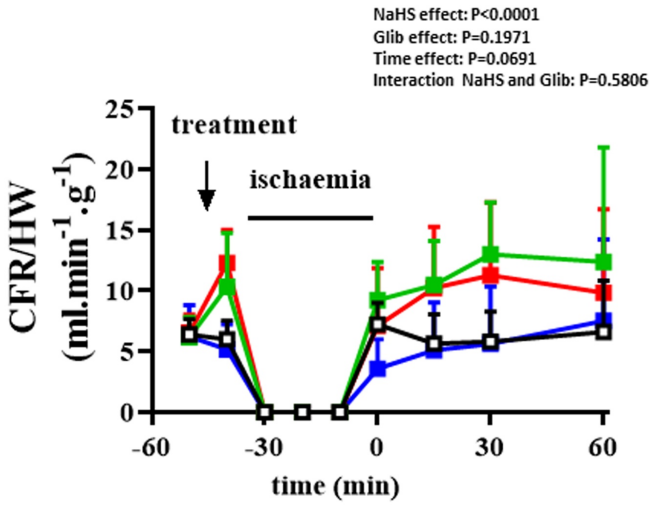

A.
NaHS effect: P<0.0001
Glib effect: P=0.4215
Time effect: P=0.0025
Interaction NaHS and Glib: P=0.0055
dP/dt max recovery (% baseline)
treatment
ischaemia
150
100
50
0
-60
-30
0
30
60
B.
NaHS effect: P=0.0043
Glib effect: P=0.4375
Interaction: P=0.0465
AUC of dP/dt max recovery during reperfusion (arbitary)
6000
4000
2000
0
C.
NaHS effect: P<0.0001
Glib effect: P=0.3546
Time effect: P=0.0030
Interaction NaHS and Glib: P=0.0029
dP/dt min recovery (% baseline)
time(min)
D.
NaHS effect: P=0.0105
Glib effect: P=0.4757
Interaction: P=0.0497
AUC of dP/dt min recovery during reperfusion (arbitary)
Con
Con +NaHS
Con +NaHS +Glib
Con +Glib

Figure 4

A.


NaHS effect: P<0.0001
Glib effect: P=0.1971
Time effect: P=0.0691
Interaction NaHS and Glib: P=0.5806
treatment
ischaemia
CFR/HW
(ml.min-1.g-1)
25
20
15
10
5
0
-60
-30
0
30
60
time (min)

B.

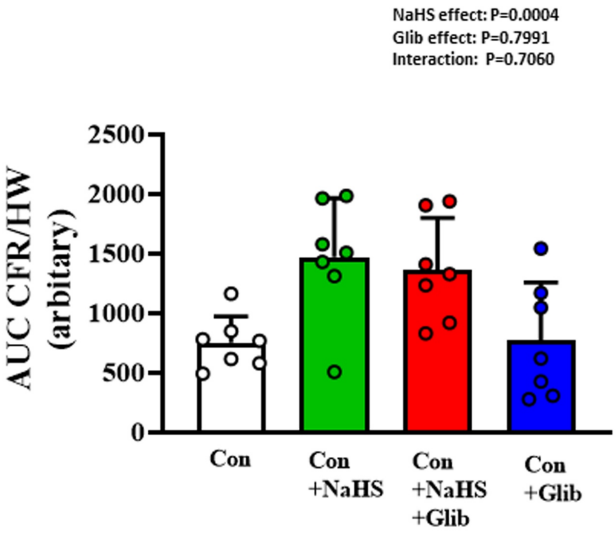

NaHS effect: P=0.0004
Glib effect: P=0.7991
Interaction: P=0.7060
AUC CFR/HW
(arbitary)
2500
2000
1500
1000
500
0
Con
Con
+NaHS
Con
+NaHS
+Glib
Con
+Glib

Figure 5

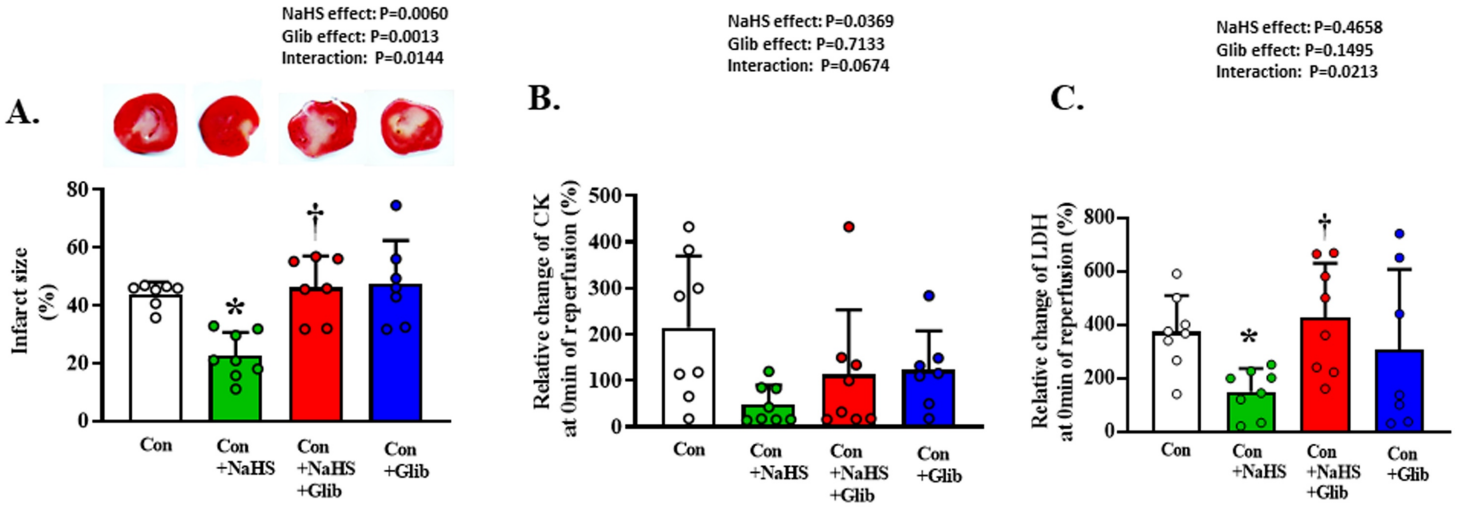

A.
NaHS effect: P=0.0060
Glib effect: P=0.0013
Interaction: P=0.0144
Infarct size (%)
0
20
40
60
80
Con
Con +NaHS
Con +NaHS +Glib
Con +Glib
*
†
B.
NaHS effect: P=0.0369
Glib effect: P=0.7133
Interaction: P=0.0674
Relative change of CK at 0min of reperfusion (%)
0
100
200
300
400
500
Con
Con +NaHS
Con +NaHS +Glib
Con +Glib
C.
NaHS effect: P=0.4658
Glib effect: P=0.1495
Interaction: P=0.0213
Relative change of LDH at 0min of reperfusion (%)
0
200
400
600
800
Con
Con +NaHS
Con +NaHS +Glib
Con +Glib
*
†

Figure 6