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 a novel technique and show the utility of the technique for exploring cardioprotective roles of H2S in the chicken embryo heart. This work lays the foundation for studying the direct effects of H2S 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 (H2S) 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 1 mM 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 area 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 coronary flow rate (CFR) during baseline (mean ± SD for AUC: 134.3 ± 91.6 vs. 92.2 ± 35.8) and post IR (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$_2$S confers embryonic cardiac protection via opening of myocardial $K_{ATP}$ channels and not via increasing CFR. H$_2$S may prove a useful therapeutic agent to protect the human fetal heart against IR injury, as may occur in complicated labour.

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### 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$_2$S is vital to cardiovascular health (Shen et al. 2015). For instance, clinical studies report that a decrease in endogenous H$_2$S 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$_2$S donor protects the adult heart from ischaemic reperfusion (IR) injury (Johansen et al. 2006). However, whether H$_2$S confers protection in the developing heart before birth remains completely unknown.

Mechanisms underlying cardiac protection by H$_2$S 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 $K_{ATP}$ channels 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 remarkably tolerant to episodes of ischaemia produced by complete occlusion of the umbilical cord, even those lasting for periods longer than 20 min. 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 (Patterson & 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 H2S confers protection against IR injury in the embryonic heart via opening of KATP channels and not via increasing coronary flow. This work lays the foundation to study the direct effects of H2S therapy on the embryonic heart independent of effects on the mother and the placaenta 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 PC6CEFE59).

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% O2, 37.9°C, 45% humidity, 12:12 h 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, MgSO4.7H2O:1.2 mM, KH2PO4:1.2 mM, NaHCO3:25 mM, glucose:10 mM, CaCl2.2H2O:1.3 mM), then mounted onto the Langendorff apparatus which was maintained at constant physiological temperature (Fig. 1). Great care needs to be taken when mounting the heart as the chicken embryo 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 O2:CO2, 95:5%) delivered at a constant pressure of 40 cmH2O 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 (LVP), 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 H2S donor; Hosoki et al. 1997), Control+Glib (glibenclamide, a non-selective inhibitor of KATP opening, Ripoll et al. 1993) or Control+NaHS+Glib. All drugs were given as a single bolus at 1 mM concentration dissolved in 0.5 ml of buffer vehicle, 10 min 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
Ten minutes after treatment, half an hour of global ischaemia was induced by shutting off the supply of
perfusate (Fig. 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 ischaemic insult (at −30 and 0 min reperfusion time).

Infarct size analysis

Following 2 h 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 × 1.5 mm-thick sections using a custom-made slicer (Fig. 2A). Mid-cardiac slices (Slice 3) were incubated with 0.1% triphenyltetrazolium chloride dissolved in a phosphate buffer (mixture of Na$_2$HPO$_4$ (77.4%) and NaH$_2$PO$_4$ (22.6%)) for 20 min at 37°C. Mid-cardiac slices were then scanned (Scanjet 300, Hewlett Packard, Cambridge, UK) and the percentage infarct size from this section was determined using Image J software (version 1.52, National Institute of Health, Bethesda, MD, USA; Fig. 2B). To quantify the infarct size, the colour threshold

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 a constant pressure of 40 cmH$_2$O, 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 basel recording, a 30 min global ischaemic challenge was introduced followed by 120 min of reperfusion. Sodium hydrosulfide (NaHS) and/or glibenclamide (Glib) were administered as a bolus at a dose of 1 mM 10 min 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.
function in ImageJ was used to filter the background and select the infarcted area (Fig. 2B–F). ImageJ then reported the number of pixels in the infarcted area and the total slice area. Infarct 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, USA), while lactate dehydrogenase (LDH) activity was assessed using a colorimetric assay (Siemens Dimension RxL analyser). Activity of these enzymes are established markers of cardiac injury (Niu et al., 2018) and were expressed as percentage 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 means ± SD. Comparisons were assessed for statistical significance using two- or three-way ANOVA. If interactions between main factors were significant, the Tukey post 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 (Fig. 3A–D), as well as cardiac contractility (\( \text{dP}/\text{d}t_{\text{max}} \)) and relaxability (\( \text{dP}/\text{d}t_{\text{min}} \)) during reperfusion (Fig. 4A–D). Administration of NaHS prior to ischaemia restored cardiac function towards basal levels post IR (Fig. 3A–D and 4A–D), with the AUC of cardiac recovery significantly greater for all four cardiac functional variables in Con+NaHS vs. controls (Figs 3B and D and 4B and D). When NaHS was co-administered with Glib, there was no longer any cardioprotective effects (Figs 3A–D and 4A–D). Treatment with Glib alone did not have any significant effect on cardiac functional recovery (Figs 3A–D and 4A–D).

Coronary flow rate
CFR normalised to chicken embryo heart weight (Table 1 and Fig. 5) did not differ between experimental groups. There was an increase in relative CFR following NaHS treatment during baseline, prior to ischaemia (Fig. 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 (Fig. 5A and B). Here, the effects of NaHS on relative CFR were not altered when co-administered with Glib (Fig. 5A and B). Treatment with Glib alone had no effect on relative CFR (Fig. 5A and B).

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**Figure 2.** Heart slicer and measurement of infarct size
A, a custom made slicer for use with the chicken embryo heart. B, 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.
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 (Fig. 6A). This beneficial effect of NaHS was blocked when NaHS was given with Glib (Fig. 6A). Treatment with Glib itself had no significant impact on infarct size (Fig. 6A).

Perfusate analysis

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

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 for isolating the direct effects of adverse conditions during development on embryonic cardiac function independent of effects on the maternal and/or

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placental physiology, highlighting a conceptual advance in the field of early origins of heart disease and developmental programming. We also provide novel evidence to support the hypothesis that H₂S has cardioprotective effects in the embryonic heart against IR injury and that the mechanism of protection involves opening of myocardial K<sub>ATP</sub> channels, independent of the vasodilator effects of H₂S 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 H₂S donor (NaHS) prior to IR almost halved the extent of infarct size. Therefore, H₂S appears to significantly protect the developing heart from infarction. Moreover, data in the present study suggest that H₂S-mediated infarct protection in the embryonic heart relies on the opening of K<sub>ATP</sub> channels, given that the NaHS-mediated reduction in infarct size is negated when co-administered with Glib, an antagonist of K<sub>ATP</sub> opening. Per fusate 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.

![Figure 3. NaHS treatment improves cardiac chronotropic and inotropic recovery after an ischaemic challenge via opening K<sub>ATP</sub> channels](image)

Values are means ± 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. 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 1 mM 10 min 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 post hoc test was used to isolate differences: *P < 0.05, Con vs. Con+NaHS; †P < 0.05, Con+NaHS vs. Con+NaHS+Glib.
resulting from IR injury. Moreover, our data suggest H₂S can protect against this cardiac injury, with values for LDH and CK significantly lower when NaHS was given prior to ischaemia. This H₂S-mediated cardioprotection appears to rely on opening of K<sub>ATP</sub> channels, since treatment with both NaHS and the K<sub>ATP</sub> 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<sub>max</sub>) and relaxability (dP/dt<sub>min</sub>) 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 H₂S translate at a functional level. Our study also suggests that H₂S-mediated protection of embryonic cardiac function depends on the opening of K<sub>ATP</sub> channels, as co-administration of NaHS with Glib prior to ischaemia diminished the functional protection conferred by NaHS.

It is established that H₂S 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 persisted 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 H₂S 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 the hypothesis that the cardioprotective effects of H₂S are due to opening of myocardial K<sub>ATP</sub> 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<sub>ATP</sub> channels.

Glibenclamide (Glib) is a member of the sulfonylurea family, which blocks K<sub>ATP</sub> channels (Luzi & Pozza, 1997; Negroni et al. 2007). In the pancreatic β-cell membrane, Glib reduces the conductance of the K<sub>ATP</sub> channel and the

**Figure 5. NaHS treatment increases coronary flow rate independent of opening of K<sub>ATP</sub> channels**

Values are means ± 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 1 mM 10 min 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 K<sub>ATP</sub> channels**

Values are means ± 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 min of reperfusion. C, relative change from baseline in the concentration of lactate dehydrogenase (LDH) at 0 min 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 1 mM 10 min prior to onset of ischaemia. Comparisons were assessed for statistical significance using two-way ANOVA. If interactions between main factors were significant, the Tukey post hoc test was used to isolate differences: *P < 0.05, Con vs. Con+NaHS; †P < 0.05, Con+NaHS vs. Con+NaHS+Glib.
reduced $K^+$ efflux determines membrane depolarization and influx of $Ca^{2+}$ that promotes insulin secretion (Lu & Poizza, 1997; Negroni et al. 2007). Consequently, Glib has been used for many years in the treatment of type II diabetes (Lu & Poizza, 1997; Negroni et al. 2007). In the adult heart, the actions of Glib have mixed 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 described Glib as anti-arrhythmic with anti-fibrillatory effects, associating these cardiac beneficial effects to a reduction in the efflux of $K^+$ induced by ischaemia (Lu & 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 of 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 2 h 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 that the opening of cardiac $K_{ATP}$ channels independent of changes in coronary flow is 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/or placental physiology. We acknowledge 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 in healthy conditions but also in development complicated by suboptimal conditions, such as with chronic hypoxia, excess glucocorticoid exposure or infection. Studies should ideally compare ex vivo with in vivo techniques to assess embryonic or fetal cardiac function, using different species, with the long-term goal of translating rational therapies to the human clinical condition.

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Additional information

Data availability statement

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

Competing interests

None declared.

Author contributions

The experiments were performed at The Barcroft Centre at The University of Cambridge. Y.N., R.H. and D.G. 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. T.G., K.B. and S.F. were involved in the conception and design of the work, data analysis and its interpretation. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

asphyxia, cardiovascular, fetus, hypoxia, ischaemia

Supporting information

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

Statistical Summary Document