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Hydrogen Sulfide Improves Cardiomyocyte Function in a Cardiac Arrest Model

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Background: Cardioplegic arrest is a common procedure for many types of cardiac surgery, and different formulations have been proposed to enhance its cardio-protective effect. Hydrogen sulfide is an important signaling molecule that has cardio-protective properties. We therefore studied the cardio-protective effect of hydrogen sulfide in cardiac cell culture and its potential therapeutic use in combination with cardioplegia formulations.

Materials/Methods: We added hydrogen sulfide donor GYY4137 to HL-1 cells to study its protective effect in nutrient starved conditions. In addition, we tested the potential use of GYY4137 when it is added into two different cardioplegia formulations: Cardi-Braun® solution and del Nido solution in an *ex vivo* Langendorff perfused rat hearts model.

Results: We observed that eight-hour pre-treatment with GYY4137 significantly suppressed apoptosis in nutrient-starved HL-1 cells (28% less compared to untreated cells; $p<0.05$), maintained ATP content, and reduced protein synthesis. In *ex vivo* experiments, Cardi-Braun® and del Nido cardioplegia solutions supplemented with GYY4137 significantly reduced the pro-apoptotic protein caspase-3 content and preserved ATP content. Furthermore, GYY4137 supplemented cardioplegia solutions decreased the S-(5-adenosyl)-L-methionine/S-(adenosyl)-L-homocysteine ratio, reducing the oxidative stress in cardiac tissue. Finally, heart beating analysis revealed the preservation of the inter-beat interval and the heart rate in del Nido cardioplegia solution supplemented with GYY4137.

Conclusions: GYY4137 preconditioning preserved energetic state during starved conditions, attenuating the cardiomyocytes apoptosis *in vitro*. The addition of GYY4137 to cardioplegia solutions prevented apoptosis, ATP consumption, and oxidative stress in perfused rat hearts, restoring its electrophysiological status after cardiac arrest. These findings suggested that GYY4137 sulfide donor may improve the cardioplegia solution performance during cardiac surgery.

MeSH Keywords: **Cardiac Surgical Procedures • Cardioplegic Solutions • Hydrogen Sulfide**

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Background

Cardiovascular surgery requires interruption of coronary flow. However, restoration of blood flow to the heart after a significant period of ischemia causes myocardial damage, a process known as reperfusion injury. Cardioplegia solutions attenuate such damage during surgical procedures [1], reducing reactive oxygen species (ROS) generation, oxygen consumption, and ATP depletion in the heart. Hyperkalemic cardioplegia formulation is the most accepted strategy to preserve heart tissue during cardiac arrest. This procedure increases the extracellular potassium concentration [2], inducing cardiomyocytes depolarization and conferring a relative myocardial protection [3].

Many molecules have been described which may improve the cardioplegia solution performance. Hydrogen sulfide (H_2S) is an endogenous gaseous mediator that exerts a broad range of physiological actions in mammalian tissues, such as participating in cellular homeostasis and protecting cells from necrosis, apoptosis, and oxidative stress [4,5]. In the heart, H_2S has protective effects on myocardial ischemia-induced cell apoptosis [6,7], whereas its reduction in a cardiac injury model increases myocardial infarct size, suggesting a role for endogenous H_2S production in preventing myocardial ischemia injury [8]. Conversely, therapeutic delivery of H_2S in a myocardial ischemia model reduces the injury size, decreases the mortality rate, improves cardiac function, suppresses inflammation, and attenuates fibrosis [9,10], suggesting a potential therapeutic role of the molecule in the heart.

Sodium hydrosulfide (NaHS) is the most common H_2S-releasing drug used to evaluate the cardio-protective effects of exogenous H_2S in experimental models. However, rapid release of H_2S in aqueous media has been observed, which does not mimic the physiological production of H_2S in tissue [11]. In contrast, the controlled release of H_2S from the GYY4137 sulfide donor drug has an important advantage to the study of their physiological effects in tissue [12]. In aqueous solution at physiological temperature and pH, the release of H_2S from GYY4137 is slow (4% to 5% H_2S at 1 mmol/L in 25 minutes) and does not cause significant cytotoxic effect [11]. GYY4137 administration has been associated with vasodilation and anti-hypertensor activity in rats, whereas no effect has been observed in the cardiac function of health hearts [11].

Given this information, we tested the hypothesis that GYY4137 modulates metabolism and reduces oxidative stress in cells, protecting cardiomyocytes from death induced by nutrient starvation, which could contribute to improve performance of cardioplegia solutions during cardiac arrest. GYY4137 protects HL-1 cardiomyocytes from death by nutrient starvation and safeguards ATP content and protein synthesis. Furthermore, the addition of GYY4137 to cardioplegia solutions in isolated Langendorff-perfused rat hearts preserved the energy state of the heart and promoted a reduction in apoptosis and oxidative stress levels. Therefore, the slow H_2S releaser GYY4137 is a potential molecule to preserve explanted tissue/organ during *ex vivo* situations.

Material and Methods

Animals

Adult male Wistar rats (300–350 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). All procedures were carried out in accordance with the Spanish Regulations for the Protection of Experimental Animals (R.D. 1201/2005; B.O.E. 21.10.2005) and were approved by institutional ethical and animal care committees.

Cell culture

HL-1 cell line, an atrial cardiac muscle cell line from the AT-1 mouse, was purchased from the ATCC (Rockville, MD, USA). Cells were cultured in complete Claycomb® medium (Sigma-Aldrich, Madrid, Spain) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM norepinephrine. GYY4137 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA; sc-224013). For GYY4137 preconditioning, HL-1 cells were incubated overnight in complete Claycomb medium with 300 µM GYY4137 or the equivalent concentration of inactive GYY4137; after which, the medium was replaced with one of the following experimental treatments: 1) complete Claycomb medium as nutrient complete medium [13] (formula is proprietary to SigmaAldrich); 2) Krebs-Henseleit (KH) medium (118.4 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $MgSO_4$, 2.44 mM $CaCl_2{\cdot}2H_2O$, 28.6 mM $NaHCO_3$, 10 mM glucose, and 10 mM Hepes, pH 7.4) which established a medium without amino acids but with glucose, mimicking a slight nutrient starvation; 3) KH without glucose to simulate stress induced by nutrient deprivation (-Glu KH); or 4) PBS to simulate an extreme nutrient deprivation in cells. All experimental treatments were supplemented or not as appropriate with 300 µM GYY4137 and all cultures were incubated at 37ºC with 5% of CO_2. To inactivate GYY4137, the control group was heated for two hours at 90 ºC and stored for one week at room temperature. Release of H_2S from GYY4137 *in vitro* is both temperature and pH dependent, with limited generation on ice (4 °C) with enhanced release with heating, as described in Li et al. [11].

Apoptosis assessment

HL-1 cells were preconditioned or treated with GYY4137 as described. The pro-apoptotic drugs staurosporine (20 nM) and

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doxorubicin (20 μM) were added to the treatments to determine whether GYY4137 protected not only by nutrient starvation but also by toxins in HL-1 cells. Apoptotic cells were identified by double-positive staining for recombinant fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide using the Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). Flow data acquisition and analysis was carried out on a Beckman Coulter FC500 MCL Flow Cytometer (Beckman Coulter, Brea, CA, USA).

Western blotting

HL-1 cells were seeded at 200,000 cells/well in a 6-well plate. At the end of treatment, cells were collected, washed with PBS, and lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannhein, Germany). For *ex vivo* experiments, frozen ventricular heart tissue was homogenized in the same lysis buffer with a Polytron homogenizer. Cells and homogenized tissue were centrifuged at 15,000 g at 4°C for 15 minutes and the protein supernatant was collected. Twenty-five micrograms of protein lysate were loaded onto each lane of a 4–12% Bis-Tris gel (NuPAGENovex, Invitrogen, Carlsbad, CA, USA). Following electrophoresis, gels were transferred to PVDF membranes, blocked in 5% non-fat milk diluted in PBS and then incubated with anti-caspase-3, anti-PARP (both at 1: 1,000; Cell Signaling Technology, Danvers, MA, USA), anti-β-tubulin or anti-actin (both at 1: 1,000; Abcam, Cambridge, UK) overnight at 4°C. After several washes, the membranes were incubated with appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1: 10,000; Invitrogen) for one hour at room temperature with shaking. Membranes were treated with luminol reagent (ECL; Pierce, Rockford, IL, USA) and exposed to x-ray film.

Nascent protein assay

Protein synthesis was measured in cells by methionine incorporation. Briefly, cells were first cultured for one hour in Dulbecco's modified Eagle´s medium without methionine (Life Technologies, Paisley, UK), containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate. Subsequently, cells were pulsed for three hours with the methionine analogue L-azidohomoalanine (AHA) (Invitrogen; c10102) under various treatments with 300 μM of active or inactive GYY4137. As a control, cycloheximide (actidione, Sigma-Aldrich) was used to inhibit protein synthesis. After treatments, cells were homogenized in PBS buffer containing 1% SDS and protease inhibitors (Roche) and an aliquot of the lysate was taken for a ligation reaction between the azide-containing AHA incorporated into nascent proteins and biotin alkyne (PEG4 carboxamide-Propargyl Biotin) (Invitrogen; B10185) using the Click-iT protein reaction buffer kit (Invitrogen; 10276). A dot-blot system was used to transfer the proteins onto a nitrocellulose membrane. Various controls were established to test the experimental system as follows: a negative control with non-biotinylated AHA, a positive control using growing HL-1 cells in complete Claycomb medium, a no-analogue control (without AHA incorporation), and an actidione control comprising HL-1 cells in Claycomb medium pre-treated with cycloheximde.

ATP quantification

HL-1 cells were seeded in 12-well plates at 10,000 cells per well in complete Claycomb medium. Cells were preconditioned with GYY4137 over 12 hours and ATP content was analyzed periodically. To evaluate the cardio-protective effect of H_2S during nutrient deprivation, HL-1 cells were preconditioned overnight with GYY4137 and the culture medium was then replaced with PBS to promote starvation. ATP content was analyzed at various times. To measure ATP, cells were washed twice with PBS buffer and then lysed with the addition of somatic cell ATP releasing reagent (FLSAR, Sigma-Aldrich). The supernatant was collected and kept at –20°C until analysis. ATP content was measured with the bioluminescent ATP somatic cells assay kit (FLASC, Sigma-Aldrich). Luminescence of triplicate samples was read on a VICTORTM X3 Multilabel Plate Reader (PerkinElmer) with a test wavelength of 570 nm. Rat hearts were explanted and stored at –80°C for ATP quantification with the colorimetric ATP Assay Kit (Abcam) following the manufacturer's instructions. Briefly, tissue samples were homogenized in ATP assay buffer, deproteinized with 1 M perchloric acid, and ATP content was determined by absorbance at 570 nm. ATP levels from experimental samples were compared to sham samples (explanted hearts without treatment).

Metabolite analysis

Frozen heart tissues (50–100 mg) were placed in 2-mL tubes containing CK14 ceramic beads (Precellys, France). Then, six volumes of N-ethyl maleimide (NEM) 50 mM in PBS were added per weight of sample (mL/g). Sample homogenization for 25 seconds at 6,000 g at 4°C was repeated twice in a Precellys 24 Dual system equipped with a Cryolys cooler, also from Precellys. Samples were centrifuged at 10,000 g for 5 minutes at 4°C and supernatants were transferred to clean tubes. Tissue extraction was repeated using four volumes of PBS containing 50 mM NEM. Finally, supernatants were pooled and stored at –80°C for further analysis. Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) was then used to determine the concentration of indirect biomarkers of oxidative stress. Reduced glutathione (GSH), oxidized glutathione (GSSG) [13], S-(5-adenosyl)-L-methionine (SAM), and S-(adenosyl)-L-homocysteine (SAH) were selected as key markers to evaluate oxidative protein damage. The UPLC-MS/MS system consisted of a Waters Acquity UPLC System (Manchester, UK) coupled to a Waters Xevo TQ-S mass

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spectrometer. Quantitative analysis was performed with Waters MassLynx 4.1 software. The analytical method has been described elsewhere [14].

Perfusion of isolated hearts

The standard Langendorff system (Harvard Apparatus) was used to evaluate the effects of GYY4137 in cardioplegia solutions. Wistar rats were heparinized (500 U/kg, i.p.) and then anesthetized with sodium thiopental (50 mg/kg, i.p.). The thoracic cavity was exposed and the heart was quickly excised and immediately immersed in ice-cold Krebs-Henseleit medium (KH) (118.4 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $MgSO_4$, 2.44 mM $CaCl_2 \cdot 2H_2O$, 28.6 mM $NaHCO_3$, 10 mM glucose, and 10 mM Hepes, pH 7.4). The aorta was cannulated and a retrograde perfusion of the heart with oxygenated KH (95% O_2 and 5% CO_2) was performed at a constant flow of 10 mL/min at 37 ºC during 15 minutes to stabilize beating. A pre-treatment pseudo-ECG was recorded to analyze basal electrical activity. After stabilization, cardioplegic arrest solutions were administered by retrograde perfusion (15 mL/Kg at 10 mL/minute) and the heart was then immersed for one hour in the cardioplegic solution at 18ºC (ischemic period). After cardioplegic arrest, retrograde perfusion of the heart was performed in the Langendorff apparatus with KH for 30 minutes to monitor the recovery of spontaneous beating. During this time, a post-treatment pseudo-ECG was recorded to analyze the electrical activity after cardioplegic arrest. After reperfusion, hearts were dissected and samples were removed for analysis.

Cardioplegic solutions

We tested two cardioplegic solutions with different formulation and administration protocols. Conventional cardioplegic solution (Cardi-Braun® maintenance solution for reperfusion) contained 8.756 mg/mL trometamol, 1.5840 mg/mL sodium citrate, 0.1976 mg/mL citric acid monohydrate, 0.1512 mg/mL sodium dihydrogen phosphate dihydrate, 1.796 mg/mL potassium chloride, and 9.86 mg/mL sodium chloride (B-Braun Inc., Barcelona, Spain). Osmolarity was 646 mOsm/L. This solution required one initial administration dose (15 mL/Kg at 10 mL/minute) and a maintenance dose (5 mL/kg at 10 mL/minute) every 20 minutes. The conventional cardioplegia solution was diluted with blood, in a 4: 1 ratio of blood: cardioplegia solution. In some experiments, GYY4137 (300 µM) was added to the conventional cardioplegia solution, (conventional + GYY4137), which was administered following the same protocol as that described for conventional cardioplegia.

Additionally, we used del Nido cardioplegia [3] (kindly donate by Hospital La Fe, Valencia, Spain). Del Nido cardioplegia solution contained 144 mg/mL sodium, 117 mg/mL chlorine, 29.2 mg/mL potassium, 156 mg/mL magnesium (in mEq/L), 0.1227 mg/mL lidocaine HCl, and 3.07 mg/mL mannitol. Del Nido cardioplegia required only one administration dose (15 mL/Kg at 10 mL/minute). Del Nido cardioplegia solution was also diluted with blood, in a ratio of 1: 4 blood: cardioplegia solution. In some experiments, GYY4137 (300 µM) was added to the del Nido cardioplegia solution: (Nido + GYY4137), which was administered following the same protocol as that described for del Nido cardioplegia. In total, four experimental groups were tested as follows: 1) conventional; 2) conventional + GYY4137; 3) del Nido; and 4) del Nido + GYY4137.

Electrical measurement

Pseudo-electrocardiogram (ECG) recordings were acquired in isolated rat hearts utilizing a single lead stainless steel unipolar electrode located in the epicardial base surface,f near to the right ventricular outflow tract. Up to 15–30 minutes pseudo-ECG tracings were acquired at a 1 kHz sampling rate using a custom preconditioning and data acquisition workstation. Briefly, low-voltage unipolar pseudo-EGM signals were amplified, band-pass filtered between 1–300 Hz and digitized with 12-bit resolution. Offline pseudo-ECG analysis of stored data was performed utilizing custom Matlab (The Mathworks Inc., Natick, MA, USA) scripts for pre-processing, visualization, and quantification of heart rate variability before and after conditioning with cardioplegic solutions. After band-pass digital filtering between 0.5V 250 Hz, baseline wander was removed using a bidirectional forward-backward digital filtering strategy. Finite differential methods and wavelet transform were used for fiducial point estimation. A total average of 4,639±802 complexes was considered in each segment (N=48 rats). Heart rate was automatically quantified using custom scripts in Matlab R2010a.

Statistical analyses

Data are represented as mean ±SEM. Comparisons between two experimental conditions were performed by unpaired Student´s *t*-test. One-way ANOVA was used for multiple comparisons followed by Bonferroni´s post hoc test for pairwise comparisons. Data analysis was performed with Excel (Microsoft Office) and statistical methods with SPSS software (IBM). Differences were considered statistically significant at $p<0.05$ with a 95% confidence interval (CI).

Results

Hydrogen sulfide preconditioning reduces cardiomyocyte apoptosis induced by nutrient deprivation

Cardioplegic arrest is a metabolism stress situation for the heart. To investigate whether H_2S protects cardiomyocytes

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from apoptosis induced by a stress situation as such nutrient deprivation using an *in vitro* approach, confluent HL-1 cells were preconditioned for eight hours with the slow-releasing H_2S donor GYY4137 (300 μM) or an equivalent amount of inactive GYY4137. After this period, the medium was replaced with KH medium with or without (-Glu KH) glucose, or with complete Claycomb medium supplemented with staurosporine (20 nM) or doxorubicin (20 μM), and apoptosis was evaluated at different time intervals by flow cytometry. In both -Glu KH (Figure 1A) and KH (Figure 1B) medium, the percentage of apoptosis was lower in cells preconditioned (pre-treated) with active GYY4137 than in cells preconditioned with inactive GYY4137. By contrast, GYY4137 had no effect on cellular apoptosis induced by staurosporine (Figure 1C) or doxorubicin (Figure 1D) in Claycomb medium, indicating that the protection mediated by GYY4137 was related to an adaptation against metabolic stress/starvation rather than protection against cardiotoxic molecules. Furthermore, when HL-1 cells were incubated in -Glu KH medium and treated directly with GYY4137, without preconditioning for eight hour, no protection from apoptosis was detected (Figure 1E), suggesting that preconditioning with H_2S was essential to reduce apoptosis. To corroborate these results, we measured the relative amount of the apoptosis markers cleaved poly (ADP-ribose) polymerase (PARP) and caspase-3 in cells by Western blotting. Cells preconditioned with active GYY4137 had lower levels of cleaved PARP and caspase-3 than those preconditioned with inactive GYY4137, both in KH and -Glu KH medium (Figure 1F), which is consistent with protection against starvation-induced apoptosis.

H_2S reduced cellular ATP levels and protein synthesis during preconditioning and attenuated their decay after starvation

Cell survival is dependent on the preservation of cellular bioenergetics. To determine whether H_2S influences cellular metabolism, cellular ATP content was measured in HL-1 cells cultured in Claycomb medium and preconditioned with GYY4137 for different lengths of time. ATP content was significantly lower in cells treated for 30 minutes with active GYY4137 than in cells treated with inactive GYY4137 (Figure 2A) and continued to decrease over a period of two hours, after which time it remained constant at approximately 50% of its original level. By contrast, ATP levels in cells treated with inactive GYY4137 remained at approximately 80% of their original levels throughout the time course (Figure 2A). Since protein turnover is an ATP-requiring metabolic process, we measured protein synthesis during GYY4137 preconditioning. After a preincubation step without methionine, HL-1 cells were pulsed for three hours with the azide-containing amino acid AHA in the presence of active or inactive GYY4137 and protein synthesis was measured using Click-iT chemistry. Protein synthesis was lower in active GYY4137-treated cells than in cells treated with inactive GYY4137 as assayed by AHA incorporation (Figure 2B), suggesting that H_2S treatment reduces protein synthesis in HL-1 cells.

We next analyzed ATP levels in starvation conditions. When HL-1 cells were pre-treated for eight hours with active or inactive GYY4137 and then starved in PBS, the initial ATP content in active GYY4127-treated cells was significantly lower than in cells treated with inactive GYY4137; however, the decline in ATP content was more rapid in inactive GYY4137-treated cells after 30 minutes of starvation (Figure 2C). Furthermore, after one hour and three hours of starvation, ATP levels in inactive GYY4137-treated cells were lower than in the active GYY4137-treated group (Figure 2C). We also examined the effects of GYY4137 for protein synthesis during nutrient starvation. Consistent with the results of ATP analysis, HL-1 cells pretreated with active GYY4137 had a lower incorporation of AHA than inactive GYY4137-treated cells after 30 minutes, but after one hour and three hours of starvation a dramatic reduction in AHA incorporation was observed in inactive GYY4137-treated cells, while cells treated with active GYY4137 maintained their incorporation of AHA (Figure 2D). Collectively, these results indicate that GYY4137 promotes the preservation of ATP content and protein synthesis at basal levels.

H_2S reduced myocardial apoptosis and preserved ATP levels in isolated hearts

Having demonstrated the metabolic cardio-protective properties of GYY4137 against stress induced by nutrient deprivation in cultured cells, we next evaluated the impact of GYY4137 in two cardioplegic solutions currently used in clinical practice: conventional Cardi-Braun® (Conv) and del Nido cardioplegia (Nido). The cardio-protective effect of GYY4137 was evaluated in isolated rat hearts secured to a Langendorff apparatus through the aorta. The scheme of the procedure is shown in Figure 3A and is described in materials and methods section. After the procedure, heart tissue was analyzed for the apoptotic marker caspase-3. Western blotting showed that the levels of cleaved caspase-3 were significantly lower in heart tissue from GYY4137-supplemented groups than non-supplemented groups, both in Conv and Nido cardioplegic solutions (Figure 3B). Furthermore, we compared the relative activated caspase-3 content in Nido and Conv cardioplegia solutions supplemented with GYY4137 to determine which protected apoptosis more efficiently (left panel in Figure 3B). We observed that hearts perfused with Nido + GYY4137 significantly reduced the activated caspase-3 amount compared to hearts perfused with Conv + GYY4137. Additionally, myocardial ATP levels were significantly higher when cardioplegic solutions were supplemented with GYY4137 (Figure 3C).

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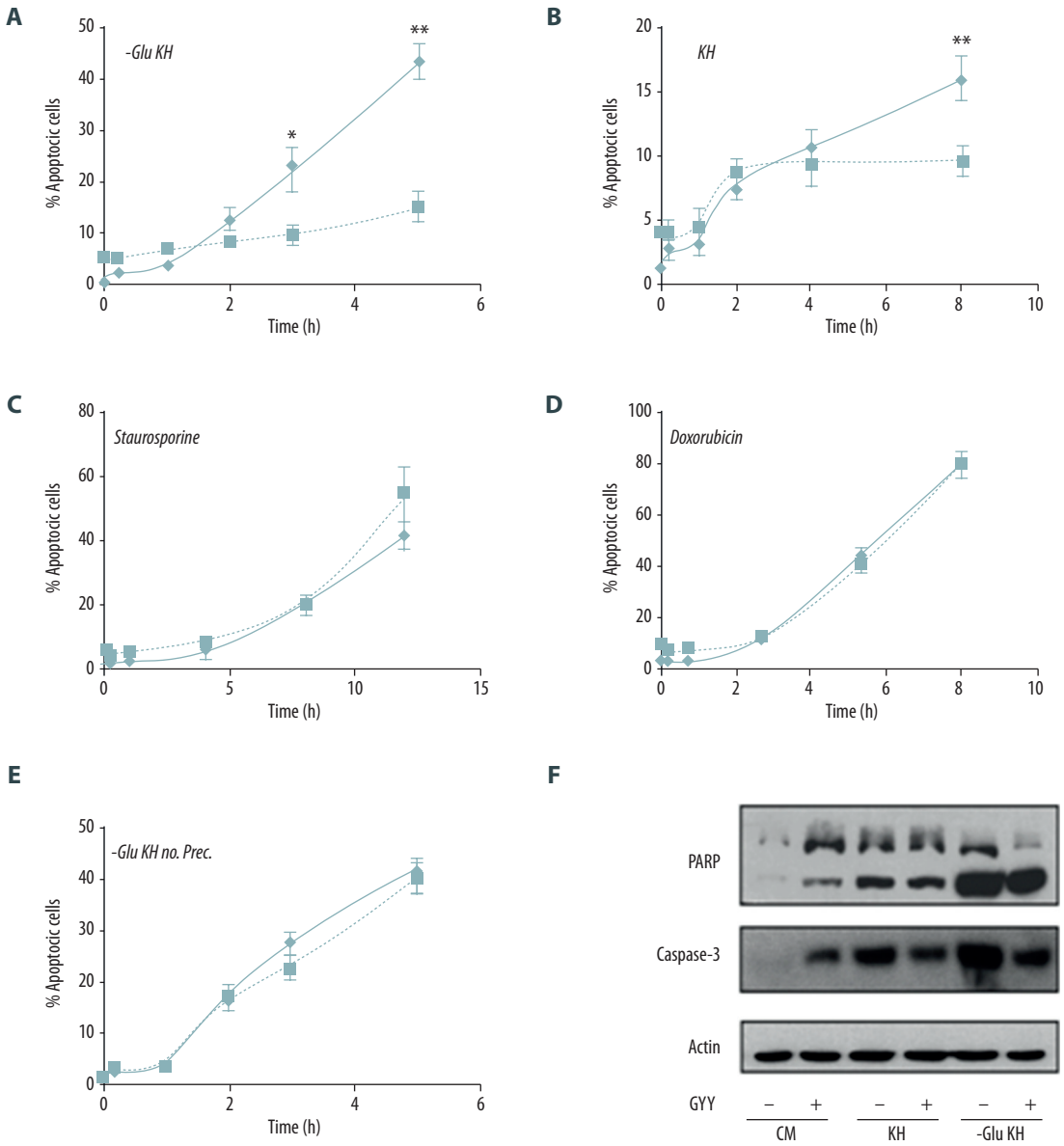

Figure 1. Effect of GYY4137 on HL-1 cell apoptosis. (**A**) Effect of GYY4137 preconditioning on apoptosis of cells cultured in glucose and amino acid deprivation medium (-Glu KH). (**B**) Effect of GYY4137 preconditioning on apoptosis of cells cultured in amino acid deprivation medium (KH). (**C, D**) Effect of GYY4137 preconditioning on apoptosis of cells treated with staurosporine (**C**) and doxorubicin (**D**). (**E**) Effect of GYY4137 on apoptosis of cells during starvation in -Glu KH medium without preconditioning. In all cases, results show percentage of apoptosis over time. Dashed lines represent cells treated with active GYY4137 and solid lines represent cells treated with inactive GYY4137. Three replicates in three differences experiments were performed per condition. * $p<0.05$, ** $p<0.01$ GYY4137 preconditioning compared to control for each time. (**F**) Western blotting for apoptotic markers (PARP and caspases-3) in preconditioned HL-1 cells with active (+) or inactive (–) GYY4137, cultured in complete Claycomb medium (CM), KH or -Glu KH medium. Actin was used as a loading control.

H_2S modulated oxidative stress levels in isolated hearts

We next analyzed oxidative stress (OS) levels in heart tissue after cardioplegic arrest and reperfusion. Since free radicals, such as ROS, are short-lived and therefore difficult to detect, an alternative strategy to assess OS is to evaluate the extent of injury triggered by their production. We therefore analyzed ROS production by measuring the presence of its oxidation target products. We measured the ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) in addition to the

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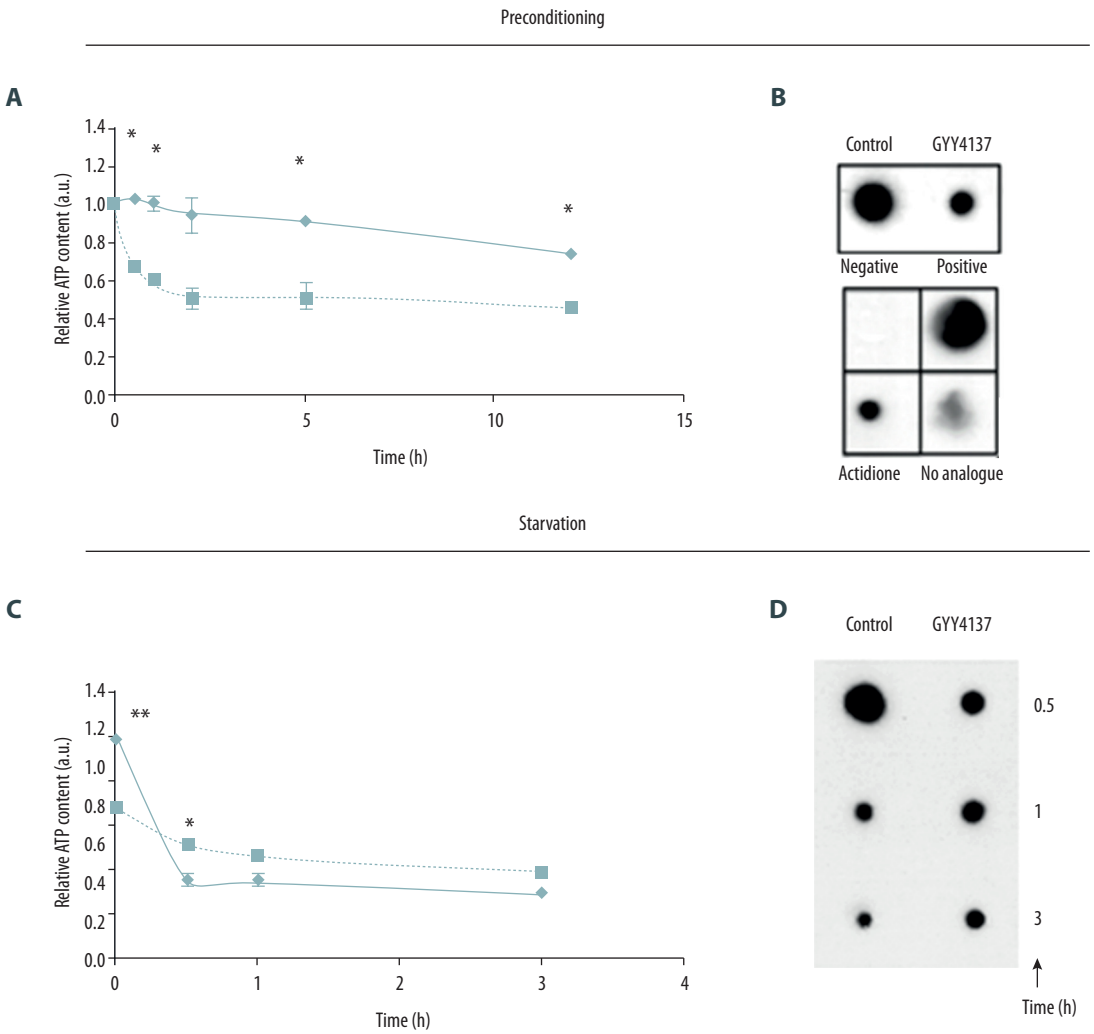

Figure 2. Effect of GYY4137 on ATP content and protein synthesis in HL-1 cells. (**A**) Relative ATP content during preconditioning. ATP content is represented as relative content to non-preconditioned cells (0 hour). Dashed line represents cells treated with active GYY4137 and solid line represents cells treated with the inactive GYY4137. (**B**) **Top panel**, dot blot assay showing methionine L-azidohomoalanine (AHA) incorporation at 3 hours of preconditioning. **Bottom panel**, experimental set up for AHA incorporation: Negative control, non-biotinylated AHA; positive control, exponential cell growth in complete Claycomb medium; no analogue, biotinylation of extract without AHA incorporation; actidione, exponential cell growth in complete Claycomb medium after pre-treatment with the protein synthesis inhibitor cycloheximide. (**C**) Relative ATP content during nutrient starvation. ATP content is represented as relative content to the initial level of control cells (0 hour). Dashed line represents cells treated with active GYY4137 and solid line represents cells treated with inactive GYY4137. (**D**) Dot blot assay showing AHA incorporation at 30 minutes (0.5 hours), 1 hour, and 3 hours after starvation. * $p<0.05$, ** $p<0.01$ GYY4137 preconditioning compared to control for each time. Triplicates were performed for each experiment (n=4).

ratio of S-(5-adenosyl)-L-methionine (SAM) and S-(adenosyl)-L-homocysteine (SAH) in hearts perfused with Conv or Nido, with or without supplemented GYY4137 (Figure 4). SAM is a precursor of GSH and an increase in the SAM/SAH ratio has been reported in cultured human lung cells exposed to high concentrations of peroxides [15]. The GSH/GSSG ratio in heart samples was increased when GYY4137 was added to either Conv or Nido cardioplegia solutions, and this was significant for Conv (Figure 4A). Moreover, the SAM/SAH ratio in both cardioplegic solutions was significantly decreased by supplementation with GYY4137, validating the protection against OS (Figure 4B). These results suggest that H_2S exerts a protective effect against OS.

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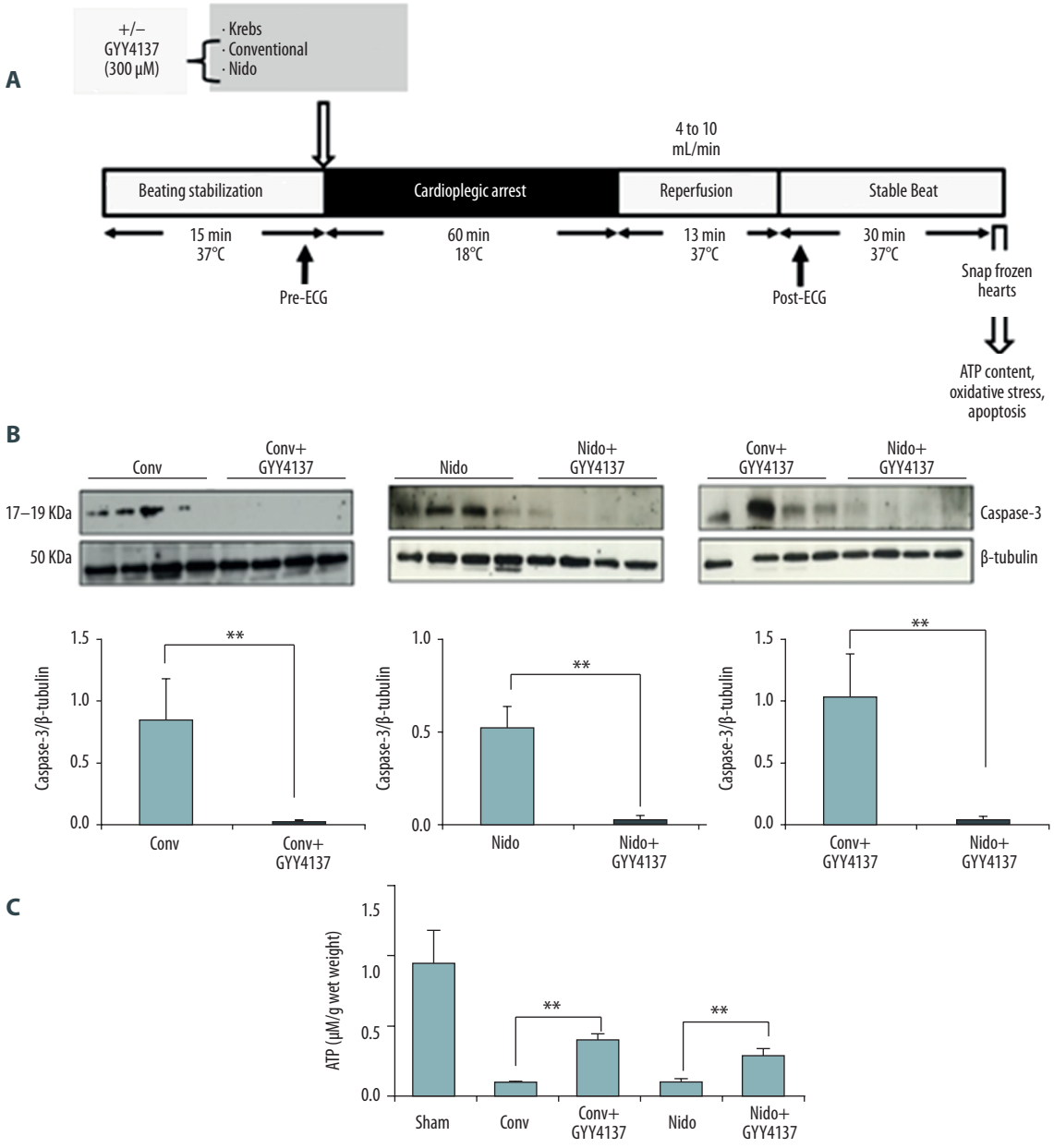

Figure 3. GYY4137 improves cardioplegic cardio-protective performance during cardiac arrest. (**A**) Scheme of the procedure to test cardioplegic solutions in rat hearts utilizing a Langendorff apparatus. (**B**) Western blotting for caspase-3 in rat heart tissues perfused with Conv or del Nido cardioplegic solutions with or without GYY4137 (n=4). The graph shows quantification of densitometry analysis utilizing ImageJ software. (**C**) ATP quantification in rat heart tissue after finalization of Langendorff perfusion protocol as described in methods section. For the sham group, rat hearts were not subjected to the experimental Langendorff protocol; the organs were directly explanted and ATP content was analyzed. * $p<0.05$, ** $p<0.01$. Four hearts were used for each experimental group.

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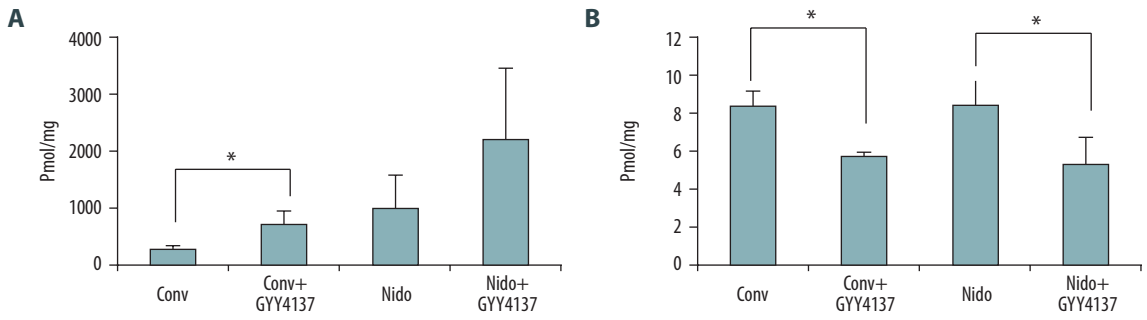

Figure 4. GYY4137 reduces oxidative stress during cardiac arrest. (**A**) Levels of oxidative stress biomarkers GSH, GSSG; and (**B**) SAM and SAH were analyzed in rat hearts treated with Conv or del Nido cardioplegia solutions with or without GYY4137. Graphs show the ratio of GSH/GSSG (**A**) and SAM/SAH (**B**). Four-six experiments were performed per condition. * $p<0.05$.

Short-term recovery of cardiac rhythm and reduction of beat-to-beat variability by H_2S

We registered electrical activation from the epicardial right ventricular base before (PRE) and after (POST) cardioplegic conditioning (Figure 5A). The overall mean cardiac frequency before treatment (N=48) was 244.31±3 0.41 bpm and was on average 8–25% lower after treatment across groups (Figure 5B). Cardiac activation of untreated hearts maintained in cold KH solution was lower (193.55±11.22 bpm, n=9) (Table 1). Neither treatment with Conv (n=10) nor 'Conv + GYY4137' (n=10) cardioplegia contributed to maintain cardiac activation since the post cardiac rate was significantly different to that of basal conditions (251.07±11.23 and 241.01±1 0.49 bpm versus 190.21±14.99 and 197.07±13.35 bpm for pre and post conditioning, respectively), although the data trend pointed to a better recovery with GYY4137-supplemented cardioplegia solution. By contrast, whereas the results for Nido cardioplegia solution alone were similar to those for Conv cardioplegia solution, 'Nido + GYY4137' conditioning preserved regular cardiac activation as shown by the non-significant change in cardiac rate after treatment (231.61±7.08 versus 212.85±7.87 bpm), indicating a prevention of short or mid-term decline in cardiac rate.

Discussion

Several studies have reported modulatory effects of H_2S in diverse cellular process of cardiomyocytes, such as mitochondria function, ROS production, or apoptosis. These actions are particularly highlighted during pathological process where hydrogen sulfide results in a cardio-protective response that preserves cardiac function [7,16,17]. In the present study, we showed that GYY4137 preconditioning in HL-1 cardiomyocytes bring under nutrient deprivation attenuated apoptosis, demonstrating the cell protective effect of H_2S in such conditions. Moreover, the reduction of ATP and protein synthesis observed in cells could underlay the mechanism of protection. In concordance, the addition of GYY4137 sulfide donor into two distinct cardioplegia solutions reduced apoptosis and preserved ATP levels in rat hearts after cardiac arrest which likely induced the recovery of cardiac rhythm and beat-to-beat variability observed in the reperfused hearts.

Hydrogen sulfide preconditioning reduced cardiomyocyte apoptosis induced by nutrient deprivation (Figure 1). By contrast, no effect on cellular apoptosis induced by staurosporine or doxorubicin was observed, suggesting that GYY4137 protection was probably related to an adaptation against metabolic stress/starvation. This potential metabolic protection is likely mediated by optimization of general metabolism under stress situation. The reduction of protein synthesis during preconditioning reduces ATP decay after starvation (Figure 2), preserving ATP levels in isolated hearts (Figure 3) and contributing to myocardial apoptosis reduction.

There is evidence that H_2S may affect cardiac mitochondria by reversible inhibition of cytochrome C oxidase, leading to a preservation of mitochondrial function [18]. In addition, H_2S has been described as a stress inductor molecule that can reduce the energy requirements of cells [19]. Therefore, it is conceivable that GYY4137 preconditioning prepares the cells for subsequent starvation period through reducing cellular energetic requirements (Figure 2A, 2B). We observed beneficial effects of GYY4137 supplementation in global heart metabolism in two different cardioplegia solutions that, we presume, wasw related to lower energetic requirements in cells maintained in GYY4137-supplemented cardioplegia. Furthermore, its protective effect was reflected in the reduction of apoptosis and oxidative stress after the ischemic period (Figures 3, 4, respectively). Interestingly, a positive role for H_2S has been reported in ischemic preconditioning (IP) [20], in which endogenous H_2S contributed to cardio-protection induced by IP through protein kinase C and sarcolemmal ATP potassium-dependent channels activity. Moreover, exogenous H_2S administration also promotes

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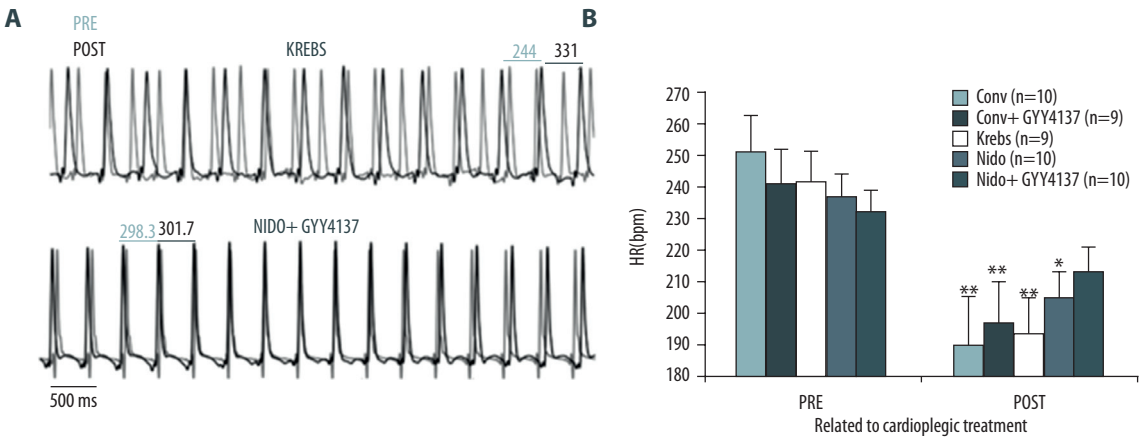

Figure 5. Short-term electrical recovery after GYY4137 treatment in the isolated Langendorff-perfused rat model. (**A**) Representative 5 second volume-conducted pseudo-ECG traces showing pre-treatment (Pre, light) or post-treatment (Post, dark) with cardioplegic solutions. A better preservation of heart rate (HR) and more regular cardiac activation is detected with del Nido+GYY4137 formulation than with untreated KH solution-preserved hearts. Traces are segmented after 15 minutes of registration from a single-lead electrode located at the epicardial base of the right ventricle in the Langendorff-perfused whole-heart. (**B**) Quantification of the effects of cardioplegic treatment on heart rate (HR, beats per minute) reveals significant slowing of cardiac activation, whereas heartrate-slowing is non-significantly different in the del Nido + GYY4137 treated group. * $p<0.01$ and ** $p<0.001$ in pre- versus post-treatment for each condition. N – number of hearts analyzed for each experimental group.

Table 1. Analysis of electrical measurement in isolated working hearts.

Cardiac parameters	KH (n=9)	Conventional (n=10)	Conventional + GYY4137 (n=9)	Nido (n=10)	Nido + GYY4137 (n=10)
Pre-treatment					
Inter-beat interval (ms)	248.48±10.02	238.96±10.88	248.95±9.84	253.61±17.25	259.05±8.71
Heart rate (bpm)	241.46±9.20	251.07±11.23	241.01±10.49	236.58±11.58	231.61±7.08
Post-treatment					
Inter-beat interval (ms)	309.99±15.99	315.43±27.08	304.45±23.10	292.77±16.73	281.88±10.74
Heart rate (bpm)	193.55±11.22	190.21±14.99	197.07±13.35	204.93±10.04	212.85±7.87
p value (inter-beat interval) Pre *vs.* Post	$p=0.0017$	$p<0.001$	$p=0.003$	$p=0.021$	$p=0.17$
p value (heart rate) Pre *vs.* Post	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.003$	$p=0.08$

Quantification of cardiac parameters extracted from volume-conducted pseudo-ECG electrical recordings in the isolated Langendorff-perfused rat heart model. One-way ANOVA and Bonferroni post-test were used to compare pre- and post-treatment. n – number of hearts used for each treatment.

cardio-protection in ischemia/reperfusion (I/R) models by inhibition of the sarcoplasmic reticulum oxidative stress [21], suggesting a similar effect that endogenous H_2S. These results support our hypothesis that GYY4137-supplemented cardioplegia solution may improve cardiac performance via protecting cardiomyocytes from metabolic decoupling, apoptosis, and oxidative stress.

Cardiac rhythm and beat-to-beat variability were restored in reperfused hearts (Figure 5, Table 1). It has been shown that GYY4137 does not alter the electrical or functional properties in healthy perfused rat hearts [11]. In contrast, its administration reduces the scar size and preserves cardiac function and structure after myocardial infarction [22], suggesting that its cardio-protective effect is only manifested during pathological process. Therefore, these findings suggest that GYY4137

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administration reduces the cardiac injury induced by the reperfusion process in our model, which results in the restoring the electrophysiological status of the heart.

In summary, H_2S preconditioning promotes a starvation-like state in normal conditions, preparing the cell for a low energetic requirement state. Such effect reduces apoptosis and oxidative stress in cardiomyocytes during nutrient deprivation conditions, improving the heart status after cardiac arrest. Further studies are warranted to test the molecular mechanism underlying to the metabolic decay induced by H_2S preconditioning in this model.

We acknowledge some limitations of our work. Although we showed the protective effect of H_2S in starved HL-1, more experiments are needed to reproduce the conditions of the *ex vivo* experiments in this work. Langendorff system allows us to take data on left ventricular pressures. In this work, for technical reasons, we could not take such measures that would have been of interest for the study.

Conclusions

In this work, we demonstrated that GYY4137 sulfide donor preconditioning reduces ATP levels and protein synthesis in cardiomyocytes, attenuating their decay and apoptosis of cells during nutrient deprivation conditions. Furthermore, the addition of GYY4137 to cardioplegic performance solutions reduces apoptosis, increases ATP levels, and prevents redox molecules oxidation in rat hearts after cardiac arrest, promoting the recovery of cardiac rhythm and beat-to-beat variability after their reperfusion. Further studies are required to test the potential therapeutic use of GYY4137 in cardioplegia solutions.

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