CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	The effect of sphingosine#1#phosphate on the endothelial glycocalyx during ischemia#reperfusion injury in the isolated rat heart
Authors	Araibi, Hala;van der Merwe, Elizabeth;Gwanyanya, Asfree;Kelly-Laubscher, Roisin
Publication date	2020-02-03
Original Citation	Araibi, H., van der Merwe, E., Gwanyanya, A. and Kelly#Laubscher, R. (2020) 'The effect of sphingosine#1#phosphate on the endothelial glycocalyx during ischemia#reperfusion injury in the isolated rat heart', Microcirculation, 27(5), e12612 (11pp). doi: 10.1111/micc.12612
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1111/micc.12612
Rights	© 2020, John Wiley & Sons, Ltd. This is the peer reviewed version of the following article: Araibi, H., van der Merwe, E., Gwanyanya, A. and Kelly#Laubscher, R. (2020) 'The effect of sphingosine#1#phosphate on the endothelial glycocalyx during ischemia#reperfusion injury in the isolated rat heart', Microcirculation, 27(5), e12612 (11pp), doi: 10.1111/micc.12612, which has been published in final form at https://doi.org/10.1111/micc.12612. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Download date	2024-04-25 03:42:54
Item downloaded from	https://hdl.handle.net/10468/10482

University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

The effect of sphingosine-1-phosphate on the endothelial glycocalyx during ischemia-reperfusion injury in the isolated rat heart

Hala Araibi[1], Elizabeth van der Merwe[1], Asfree Gwanyanya[1], Roisin Kelly-Laubscher[1,2,3].

1. Dept. Human Biology, Faculty of Health Sciences, University of Cape Town, Observatory, Cape Town, South Africa

2. Dept. Biological Sciences, Faculty of Science, University of Cape Town, University of Cape Town, Rondebosch, Cape Town, South Africa

3. Dept. Pharmacology & Therapeutics, The College of Medicine and Health, University College Cork

Running title: S1P & the glycocalyx in ischemia reperfusion

Funding:

Higher Education Ministry of Libya

National Research Foundation of South Africa: 85768

South African Medical Research Council: MRC SIR Grant No 2984

Corresponding Author: Roisin Kelly-Laubscher, email:

Rokelly81@gmail.com

ABSTRACT

Objective: Sphingosine-1-phosphate (S1P) is a natural metabolite that is cardioprotective, but its effects on endothelial glycocalyx damage during ischemia-reperfusion (I/R) are unknown. Therefore, we investigated the effect of S1P on the endothelial glycocalyx during I/R.

Methods: Isolated hearts from Wistar rats were perfused on a Langendorff system with Krebs-Henseleit buffer and pre-treated with S1P (10 nM) before I/R. Infarct size was measured by triphenyl tetrazolium chloride staining ($n \geq 6$ per group). Cardiac edema was assessed by calculating total water content (n=7 per group) and histologically quantifying the interstitial compartment ($n \geq 3$ per group). The post-ischemic coronary release of syndecan-1 was quantified using ELISA. Syndecan-1 immunostaining intensity was assessed in perfusion-fixed hearts ($n \geq 3$ per group).

Results: Pre-treatment with S1P decreased infarct size in isolated hearts subjected to I/R (P=0.01 vs. I/R). However, S1P had no effect on syndecan-1 levels in the coronary effluent or on the intensity of the syndecan-1 immunostaining signal in cardiac tissue. Heart total water content was not significantly different between control and ischemic groups but was significantly decreased in hearts treated with S1P alone.

Conclusion: These results suggest that S1P-induced cardioprotection against I/R injury is not mediated by the maintenance of syndecan-1 in the endothelial glycocalyx.

Keywords: S1P, glycocalyx, ischemia reperfusion, myocardial infarction, cardiac edema.

Abbreviations:

DMSO: dimethylsulphoxide

I/R: ischemia reperfusion

LVEDP: left ventricular end diastolic pressure

LVP: left ventricular pressure

LVSP: left ventricular systolic pressure

NFR: Net filtration rate

PFA: paraformaldehyde

RPP: Rate pressure product

S1P: Sphingosine-1-phosphate

TTC: Triphenyl tetrazolium chloride

Introduction:

Ischemic heart disease is a leading cause of deaths in many countries, ahead of other non-communicable diseases such as cancer, stroke and respiratory diseases. [1] Ischemia can cause cardiac tissue infarction and cell damage. Paradoxically, although reperfusion of ischemic tissue is important to improve the survival of the tissues and prevent irreversible cellular injury, it may lead to more cellular damage than that caused by ischemia alone, a phenomenon commonly referred to as ischemia/reperfusion (I/R) injury. [2] Although I/R injury is mainly characterized as a disease of cardiomyocytes, the coronary vasculature is a major target of the injury. [3]

Coronary vessels are lined by endothelial cells which play a key role in maintaining vascular tone, regulating hemostasis, and regulating inflammatory processes. The luminal surface of these endothelial cells is covered by the glycocalyx, a thin carbohydrate-rich layer. The endothelial glycocalyx is approximately 0.2 – 0.5 μm thick in small capillaries and becomes thicker as the vascular diameter increases. [4,5] The glycocalyx is composed of glycoproteins, proteoglycans and soluble components. [6] Proteoglycans consist of negatively charged sulphated glycosaminoglycans, such as heparan sulphate, attached to core proteins such as syndecans.

The glycocalyx provides essential functions necessary for vascular homeostasis. It regulates vascular permeability, [4,7] acts as a physical barrier between the cell adhesion molecules and blood cells, [8] and it acts as a mechanotransducer which converts shear stress of blood flow into shear-dependent endothelial responses. [5,9-11] The glycocalyx functions are dependent on the structural integrity of the glycocalyx

and the health status of blood vessels. However, the glycocalyx is very delicate and is easily damaged during I/R injury, [12] resulting in increased vascular permeability.

Sphingosine-1-phosphate (S1P) is a membrane phospholipid metabolite that has been shown to protect the heart against I/R [11,13,14], and protects against shedding of the glycocalyx after removal of plasma albumin in rat fat pad endothelial cells [15], during trauma-hemorrhagic shock, [16] during hemorrhagic shock and resuscitation [17] and during tumour cell adhesion. [18] Its concentration in the plasma and serum ranges from 0.3 - 0.5 μM and approximately 90% is bound to serum proteins, mainly high-density lipoproteins, serum albumin and low-density lipoproteins. [19] These proteins carry S1P to the endothelial cells to maintain vascular permeability. [20] Furthermore, S1P can regulate and induce synthesis of glycocalyx components after their shedding as previously reported in rat fat-pad endothelial cells. [21,22] However, this ability of S1P to stabilize and replenish the endothelial glycocalyx has not been shown in a whole organ model in the context of I/R injury. Therefore, we hypothesize that S1P-induced cardioprotection, at concentrations previously shown to decrease infarct size, is mediated by modulation of the glycocalyx during I/R in the isolated rat heart.

In this study we use the well characterized Langendorff perfused rat heart [23,24] to assess the effect of S1P on one particular glycocalyx component, syndecan-1, during an ischemia reperfusion insult. These experiments will incorporate the changes to the Langendorff perfusion system, used by other groups in the guinea pig [25-27], which allow collection of the coronary effluent for measurement of glycocalyx components. This model has proven to be an effective model previously for the assessment of a compounds ability to protect the endothelial glycocalyx during ischemia reperfusion [25,26].

Materials and Methods

Animals

Adult male Wistar rats (weight 250 - 300 g) were used in the present study. All experimental procedures were approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (Protocol AEC REF NO: 014/012) and were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Research Council, National Press 2011). Animals were obtained from University of Stellenbosch Animal unit and housed under standardized conditions (12-hour light-dark cycle and temperature of ~23 ^{o}C) at an animal facility at the University of Cape Town. Animals had access to food and water ad libitum.

Heart Isolation and Langendorff Perfusion

Hearts were isolated and perfused on a Langendorff system. Briefly, rats were anaesthetized with sodium pentobarbitone (70 mg/kg, i.p.) and injected with heparin (500 IU, i.p.). Hearts were excised rapidly, arrested in cold Krebs-Henseleit buffer (K-H) (4^{o}C), and mounted on the Langendorff retrograde perfusion system via cannulation of the aorta. The hearts were perfused with K-H buffer (comprised of mM 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 11 glucose, pH 7.4), and equilibrated with 95% O_2: 5% CO_2 throughout the protocol at constant pressure (74 mmHg) and temperature (37 ^{o}C).

Hemodynamic parameters were measured using a pressure transducer (MLT0699, ADInstruments, Australia) connected to a water-filled balloon which was inserted into

the left ventricle. This transducer was connected to a Power Lab 4/30 data acquisition system (ADInstruments, Australia) via a Bridge Amplifier (ML221, ADInstruments, Australia). The data were recorded and digitized using Lab chart software version 7 (ADInstruments). The left ventricular end diastolic pressure was adjusted between 4 and 12 mmHg at the beginning of the protocol. The pulmonary artery was cannulated to allow for measurement of coronary flow rate and collection of coronary venous effluent. Coronary venous effluent was stored for measurement syndecan-1 levels.

Treatment Protocol

All hearts were stabilized for 20 minutes and divided according to treatment and induction of global ischemia. Four groups were used (N≥ 16 per group) as described in Figure 1: Control group (n=16); hearts were infused with the vehicle DMSO (1:10000 diluted in K-H) for 7 minutes, and then perfused with K-H for 90 minutes. I/R group (n=17); hearts were infused with DMSO for 7 minutes, followed by 10 minutes washout with K-H buffer, [11,13] thereafter exposed to 20 minutes global ischemia followed by 60 min reperfusion. I/R +S1P group (n=16); Hearts were infused with S1P (10 nM) for 7 minutes, followed by 10 minutes washout, exposure to 20 minutes global ischemia followed by 60 min reperfusion. S1P group (n=17); Hearts were infused with S1P (10 nM) for 7 minutes followed by perfusion with K-H for 90 minutes.

An infusion pump (Graseby 2100, Medical Smith, UK) was used to infuse S1P and DMSO into the heart through a 3-way tap connected to both the aortic cannula and K-H reservoir. The infusion speed was adjusted to 1/10 of the coronary flow to minimise alteration of the main perfusion inflow.

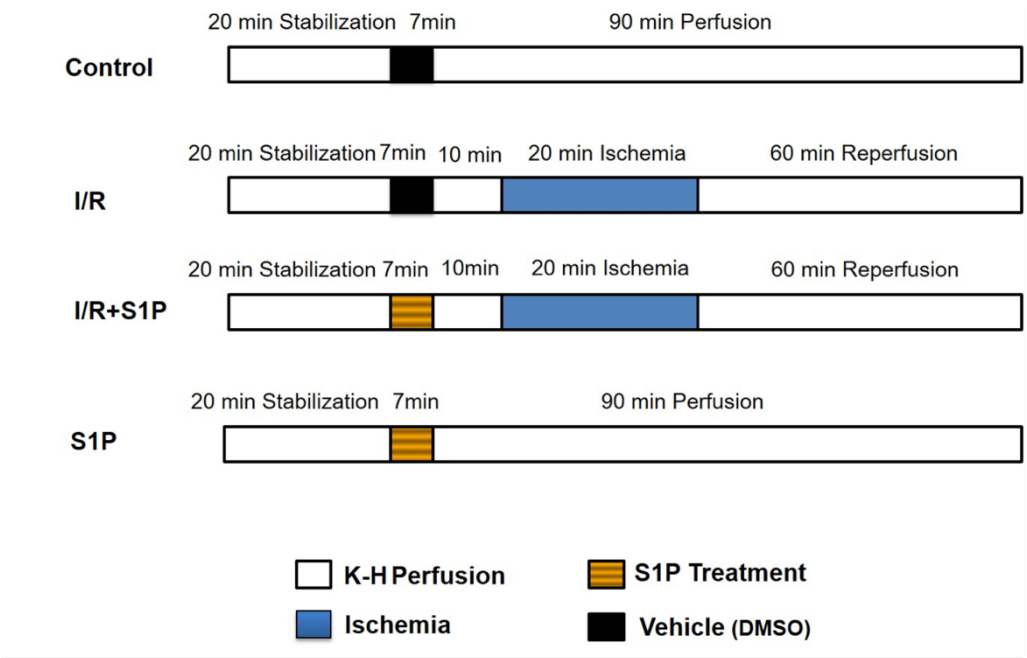

Figure 1: **Schematic representation of the Langendorff perfusion protocol.** I/R= Ischemia-reperfusion, S1P= sphingosine-1-phosphate, DMSO=dimethylsuphoxide, KH= Krebs Henseleit

Hemodynamic Measurements

Various cardiac parameters were measured throughout the experiment, including left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), heart rate, coronary flow and left ventricular developed pressure (LVDP: the difference between left ventricular systolic and left ventricular end-diastolic pressure). Rate pressure product (RPP) was calculated as LVDP × heart rate, and functional recovery was expressed as a percentage of baseline. The coronary flow rate was measured by timed collection of coronary effluent through cannulation of the pulmonary artery. The transudate was collected from the apex of the heart and net filtration rate (NFR) was calculated as the ratio between the transudate and effluent flow rate.

Infarct Size Analysis

In line with other studies showing S1P induced cardioprotection [11,13,14], at the end of the perfusion protocol, infarct size was determined as described previously [11]. Briefly, hearts (n≥ 6 per group) were frozen and sectioned into slices of approximately 2 mm from apex to base and the slice nearest the base of the heart was omitted. Slices were incubated in 1% Triphenyl tetrazolium chloride in phosphate buffer at 37 ^{o}C (pH 7.4) for 20 minutes. Slices were fixed in 10% formalin solution overnight. Heart slices were laid out in order between two glass sheets and digitally scanned on a flatbed scanner (CanoScan LDE 110, Vietnam). Total tissue area and infarct area were measured using ImageJ software, [28] as adapted from Shakkotai [29]. Briefly, using the polygon selection function of the software, total ventricular area (area at risk) was measured by trimming and cutting the unwanted segments including the LV cavity and anything outside the ventricles, and then tracing the outline of the slices. Once the outline was traced, it was possible to select everything inside the tracing and measure the total area of the ventricle using the "measure" tool. Then a Red-Green-Blue split function was performed. The green channel was used to highlight the infarcted tissue and the cumulative infarcted area was obtained by selecting the highlighted infarcted tissue and again using the "measure" tool. Infarct size was expressed as a percentage of the total ventricular area.

Assessment of Cardiac Edema

After 30 minutes reperfusion, hearts (n=7 per group) were weighed to obtain wet weight and weighed again after 24 hours drying in the oven at 60 ^{o}C to obtain dry

weight. To quantify formation of edema, these values were used to calculate total water weight (TWC) per gram of dry tissue weight using the formula below;

TWC = (wet weight-dry weight)/dry weight.

Extracellular edema was quantified by histological investigation.

Quantification of the Glycocalyx Components in Cardiac Effluent

In all groups, samples of coronary effluent were collected through the pulmonary artery over 10 minutes after the onset of reperfusion and used for assessing shedding of syndecan-1 ($n \geq 7$). Samples were prepared as previously described by Annecke *et al.*[25] Briefly, samples of 2 ml each were concentrated to 150 – 200 µL with 10 kDa cut-off ultrafilters centrifugal unit (Millipore, Billerica, MA, USA). Samples were aliquoted (50 µL) and used to determine syndecan-1 by ELISA (Diaclone research, Besancon, France).

Histological Investigation

Hearts from a subset of the animals (n=3 per group) were perfusion-fixed immediately at the end of the treatment protocol with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 10 min at room temperature using an infusion pump (Graseby 2100, Medical Smith, UK). Thereafter they were immersion-fixed in 4%PFA in PBS overnight at 4 ºC and were processed to wax. For immunostaining of syndecan-1, paraffin embedded sections (6 µm thickness, 3 sections/heart) were mounted on coated microscope glass slides, deparaffinised, rehydrated to water, washed in PBS and immunoblocked in % BSA in PBS/ 0.1% Triton X-100 for 2 hours at room temperature. Sections were incubated in rabbit monoclonal [EPR6454] syndecan-1 primary antibody (ab216458,

Abcam 1:500) overnight at 4° C, washed in PBS/ 0.1% Tween-20 and incubated in goat anti-rabbit HRP- linked secondary antibody (ab6721, Abcam 1:250) for 1 hour at room temperature. Sections were incubated in Vector DAB substrate (DAB Peroxidase kit, Cat.No.SK-4100, Vector, Burlingame, CA) for 6 minutes (based on prior optimisation of the DAB signal), dehydrated, cleared and mounted with Dibutyl phthalate-DPX (Sigma). Negative controls were performed by excluding the primary antibody only or excluding both antibodies to determine any non-specific cross-reactivity with the secondary antibody or endogenous peroxidase respectively.

Tile scans of each section were captured using a 40X objective lens on a Virtual Slide Microscope System (VS120-L100-W, Olympus, Japan). The left ventricular area was identified on the tile-scanned images and 20 blood vessels per section x 3 sections per heart (arteries and veins excluding small capillaries) were visually scored to assess the relative staining intensity of syndecan-1. The scoring system to evaluate the presence of syndecan-1 was based on a modification of that used by Kliment *et al.*[30] from a score of 0 - 2 to that of 0 - 3 for this study (viz. staining intensity was scored as 0 = no staining, 1 = mild staining, 2 = moderate staining, 3 = intense staining) (Figure 2) .

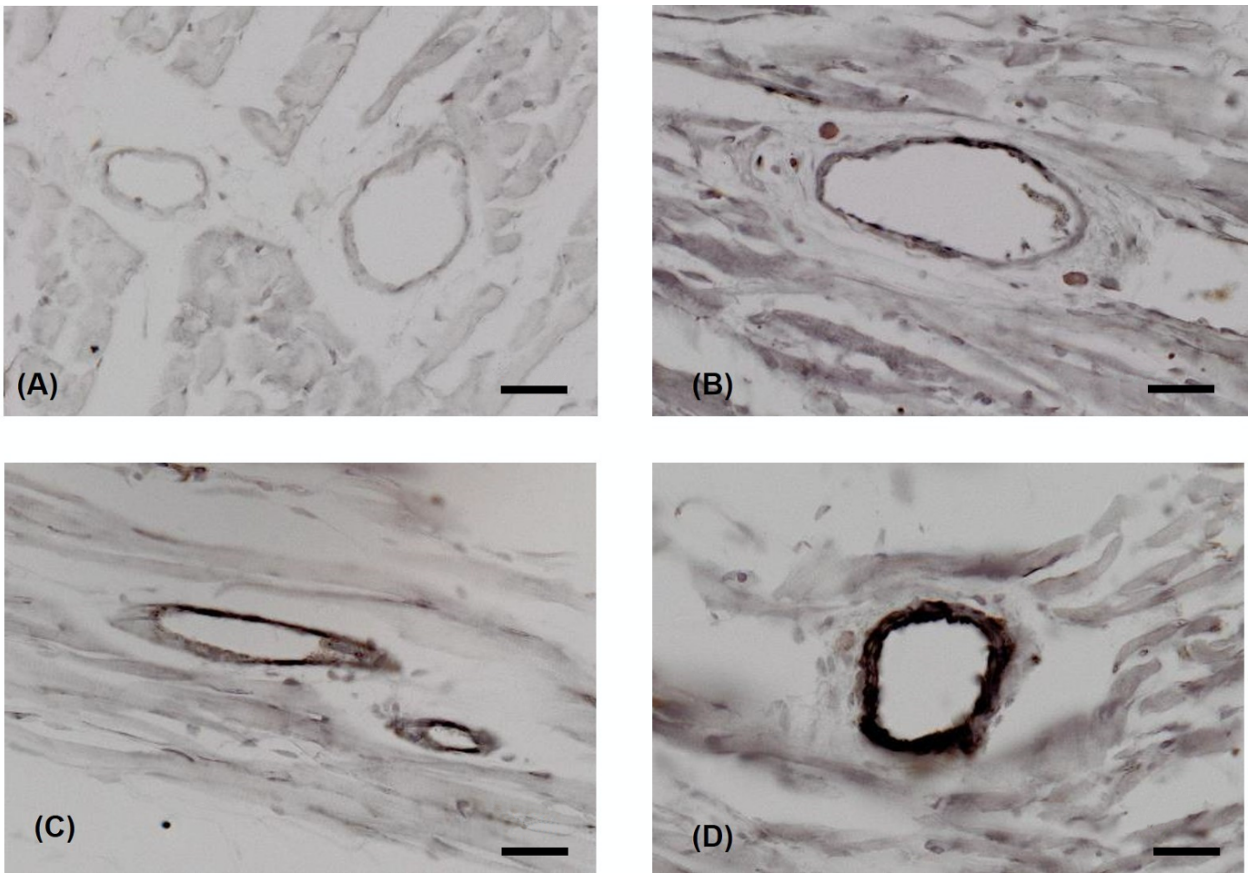

Figure 2*:* **Scoring of syndecan-1 immunostaining.** Scoring categories according to staining intensity of the vessel wall are shown as described in image analysis section (bar = 50μm); (A) Score 0 = no staining, (B) Score 1 = mild staining, (C) Score 2 = moderate staining, (D) Score 3 = intense staining .

Paraffin sections (5 μm thickness (3 sections/heart)) were stained with Haematoxylin and Eosin (H&E) for histological analysis of cardiac edema. Sections were viewed and photographed using the 20X objective lens on a Zeiss Axioskop 2 Mot plus light microscope (Carl Zeiss, Germany), fitted with the AxioCamHR camera controlled by Axiovision 4.7 software (Carl Zeiss, Germany). Images from three regions of the left ventricle (as shown in Figure 3 A) of each section (3 sections/ heart) were analysed. The extracellular compartment (i.e. any space outside the cardiomyocytes) was measured by thresholding (Figure 3 B and C) using ImageJ software [28] and the data expressed as a percentage of total area of the field of the image.

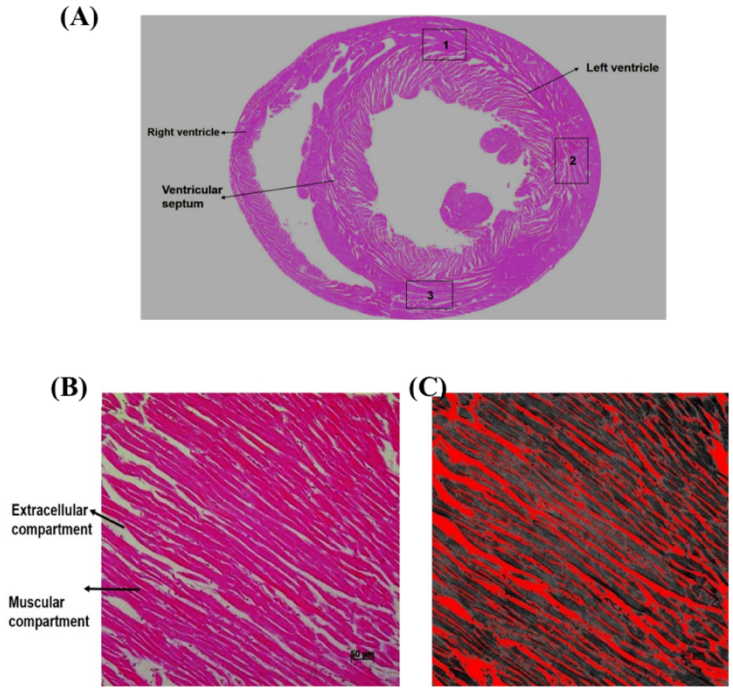

Figure 3: **Histological Analysis of Oedema:** A) Hematoxylin and Eosin(H&E)-stained paraffin-embedded section from one heart. Boxes 1, 2 and 3 represent the site of images captured (bar = 500μm). (B) Higher magnification of the H&E-stained section of selected region (as shown in A) of the left ventricle of one heart(bar = 50μm), and (C) is the same image as (B) showing thresholding of the extracellular space (red) using ImageJ software (bar = 50μm).

Statistical Analysis

All data were presented as the mean ± SEM. Data was subjected to Shapiro-Wilk normality tests. Comparisons between multiple groups were performed using one-way ANOVA, and repeated measures ANOVA were used for measurements in a

single group measured more than one time. Post-hoc tests were performed using Tukey's test. A value of $P<0.05$ was considered to be statistically significant. GraphPad Prism statistical software version 7 was used to conduct the analysis (GraphPad Prism, USA). For the semiquantitative analysis of ordinal data of relative Syndecan-1 staining intensity, no statistical analysis was done and only the pattern shown by the data is referred to.

Results

Effect of S1P on Infarct Size

Rat hearts exposed to the ischemic protocol alone had significantly increased infarct sizes (32.34 ± 4.08%, n=7) compared to control hearts (17.33 ± 1.59%, n=6, P= 0.04, control vs. I/R) and hearts treated with S1P alone (16.06 ± 4.07, n=7, P=0.02, S1P vs. I/R). Treatment with S1P before ischemia (I/R+S1P) significantly decreased infarct size to 14.17± 4.35 (P= 0.01, n=6, I/R+S1P vs. I/R) (Figure 4).

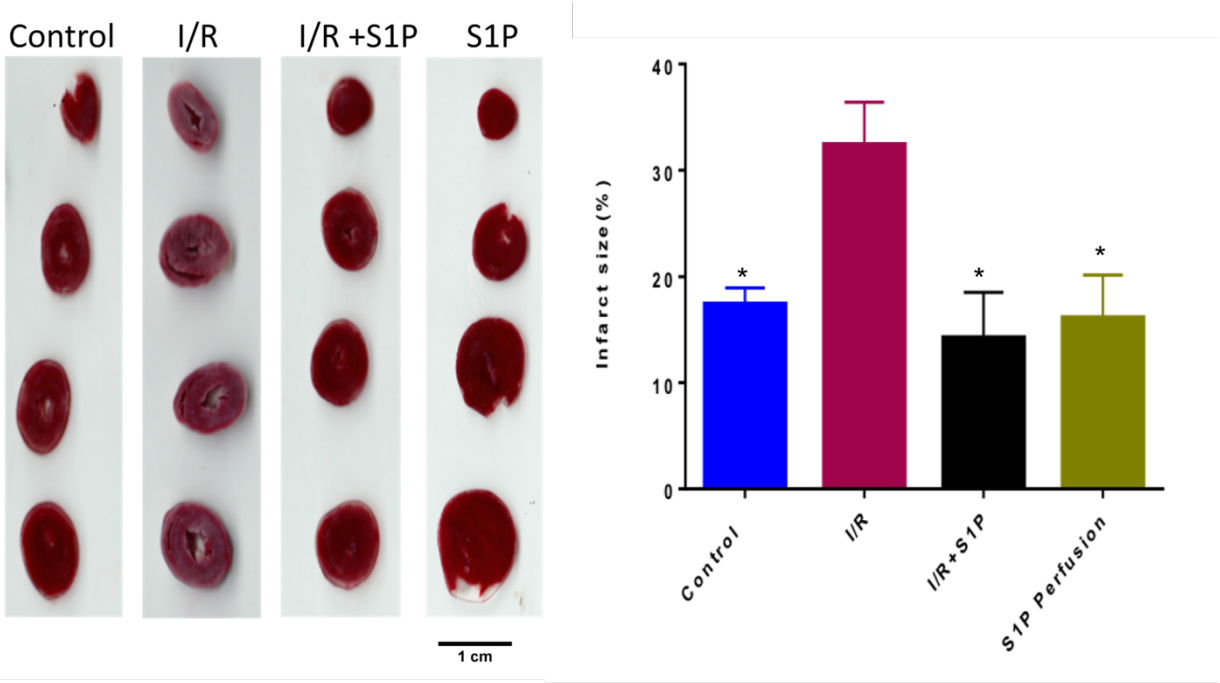

Figure 4: **Effect of S1P on infarct size of isolated hearts subjected to 20 minutes ischemia and 1 hour reperfusion**: (A) Representative images of cross sectional slices of the ventricular myocardium stained with TTC. Slices from one heart per group are shown. Since TTC stains live tissue red, infarct is seen as paler tissue. (B) Infarct size is expressed as a percentage of total ventricular area (*P<0.05 vs. I/R. N≥6/

group). I/R= Ischemia-reperfusion, S1P= sphingosine-1-phosphate, TTC= Triphenyltetrazolium chloride

Effect of S1P on Hemodynamic Parameters in Isolated Perfused Hearts

The hemodynamic data of the isolated hearts subjected to 20 minutes ischemia and 60 minutes reperfusion, with or without S1P treatment, were recorded at different time points during the protocol for all groups (Figure 5). At the end of the protocol, control hearts had postperfusion LVDP of 40.66 ± 2.02 mmHg and LVEDP of 26.15 ± 3.82 mmHg. I/R caused a significant decrease in LVDP 18.19 ± 4.78 mmHg, ($P=0.02$ vs. control) and an increase LVEDP of 61.37 ± 5.32 mmHg ($P<0.0001$ vs. control). S1P treatment before ischemia did not improve recovery of LVDP (15.51 ± 2.06 mmHg vs. I/R, $P>0.05$ n.s) or LVEDP (69.49 ± 3.62 mmHg vs. I/R, $P>0.05$ n.s). In S1P treated hearts that were not subjected to ischemia, LVDP and LVEDP were not significantly different from the control (LVDP: 39.83 ± 4.24 mmHg $P=0.99$ vs. control; LVEDP: 12.98 ± 1.86 mmHg, $P=0.1$ vs. control). Heart rate, coronary flow, net filtration rate, and RPP were not significantly different between the groups at the end of reperfusion.

The baseline hemodynamic parameters measured at the end of 20 minutes stabilization were not significantly different between the groups. However, in every single group there was a significant decrease in LVDP and CF by the end of reperfusion compared to the values measured at the end of stabilization ($P<0.0001$ at the end vs. baseline). All groups except the S1P perfusion group showed a significant increase in LVEDP by the end of reperfusion, compared to the baseline values

(P<0.001). HR was not significantly different at the end of perfusion in comparison to the baseline values in each group (P>0.05). The control and ischemia alone groups demonstrated a significant increased net filtration rate by the end of reperfusion compared to baseline (P<0.05). Similar increases were not seen for the S1P treated groups. Also, the ischemic groups (I/R, I/R+S1P) had a significant difference in net filtration rate early after reperfusion, compared to the S1P perfusion group (P≤0.002) (Figure 5E).

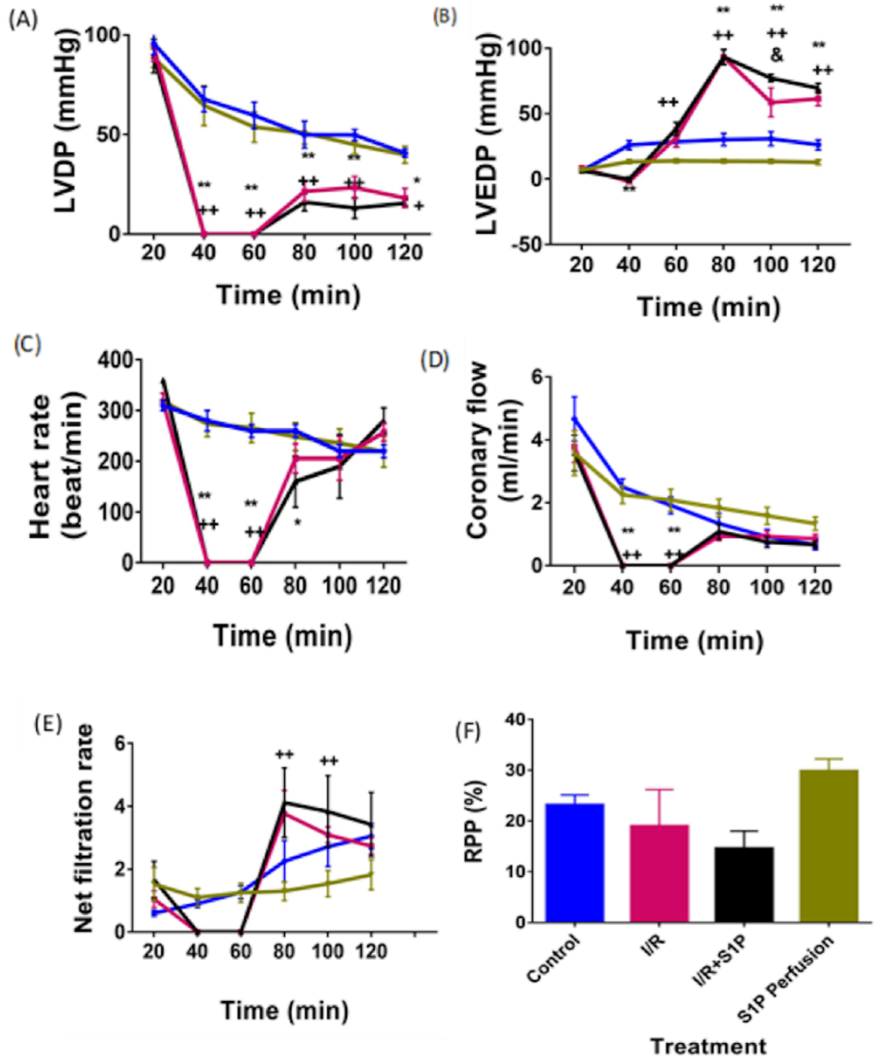

Figure 5: **Effect of S1P on hemodynamic parameters of isolated hearts for all groups.** Graphs A-E show the changes in (A) left ventricular developed pressure (LVDP), (B) left ventricular end diastolic pressure (LVEDP), (C) heart rate (HR), (D) coronary flow (CF), (E) net filtration rate (NFR) over time in each group and (F) shows rate pressure product (RPP) for each group calculated after 60 minutes reperfusion and expressed as percentage of the baseline. Control group (, blue, n=6), Ischemia/reperfusion group(I/R , red, n=7), Ischemia/reperfusion +S1P (I/R+S1P , black,n=6), S1P perfusion (, green, n=7).* P<0.05, **P<0.01 vs. control. +P<0.05, ++P<0.01 vs. S1P perfusion & P<0.05 I/R vs. I/R+S1P.

Effect of S1P on Cardiac Edema in the Isolated Perfused Heart

Mean TBW of isolated hearts were measured after 20 minutes ischemia (or equivalent perfusion for control groups) and 30 minutes reperfusion with or without S1P treatment.

Mean TBW in control hearts was 7.24 ± 0.09. The ratio was not significantly different for either of the ischemic groups (Ischemia: 6.61 ± 0.20, Ischemia +S1P: 6.76 ± 0.21, P>0.05 vs. Control), however S1P treatment alone caused a significant decrease in the ratio (6.41 ± 0.28, P=0.04).

The ratio of interstitial area (i.e. excluding cardiomyocytes) to the total area of the image field was measured to quantify cardiac edema. In the control hearts, the interstitial area was 37.06 ± 0.95 % and increased in the ischemic group (43.48 ± 1.07 %). Although not tested for significance, treatment with S1P before ischemia tended

to decrease interstitial area to control levels (37.43 ± 0.96 %). S1P treatment without ischemia had no effect on the interstitial area (37.96 ± 1.53 %) (Figure 6).

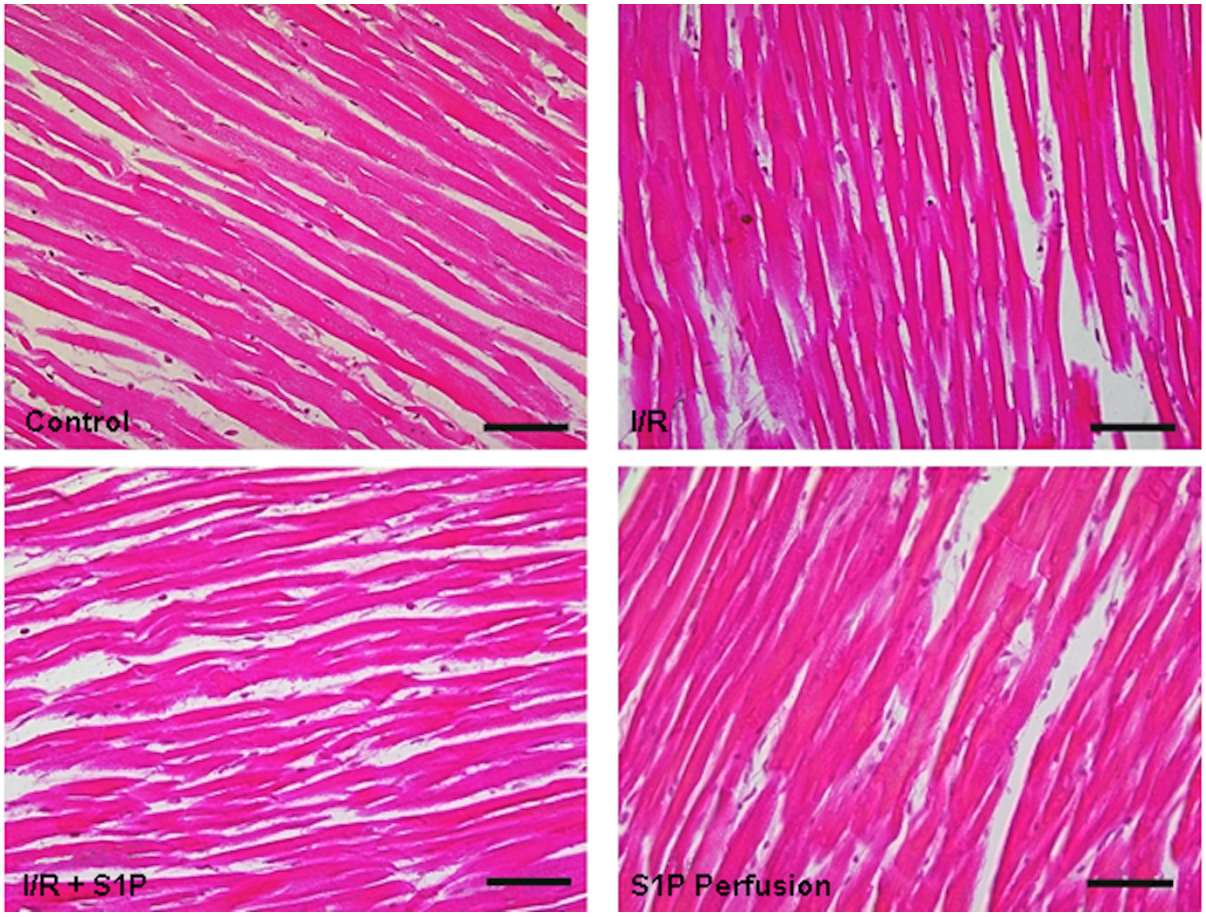

Figure 6: **Cardiac edema in H&E-stained wax sections of the left ventricle.** Morphological features of the cardiac tissue show a distinct increase in extracellular edema in the I/R group. One section from a heart in each group is shown. *(bar = 50μm).* I/R= Ischemia-reperfusion, S1P= sphingosine-1-phosphate. H&E= Haemotoxylin & Eosin

Effect of S1P Treatment on Syndecan-1 Shedding

Shedding of the glycocalyx was assessed by measuring the level of syndecan-1 in the coronary effluent using ELISA. Syndecan-1 levels in the coronary effluent of the hearts subjected to 20 minutes global ischemia followed by reperfusion (10.22 ± 1.39 ng/ml, n=10) were significantly higher than that detected in the control group (3.92 ± 0.76 ng/ml, P=0.0002, n=10). Pretreatment with S1P before ischemia had no significant effect on the shedding of syndecan-1 (8.59 ± 1.14 ng/ml, P=0.7 vs. I/R, n=7). The group treated with S1P without ischemia had similar syndecan-1 levels to the control group (3.24 ± 0.47 ng/ml, P=n.s vs. control, n=9) and was significantly different from the ischemic groups (P<0.0001 vs. I/R, P=0.0056 vs. I/R+S1P) (Figure 7A).

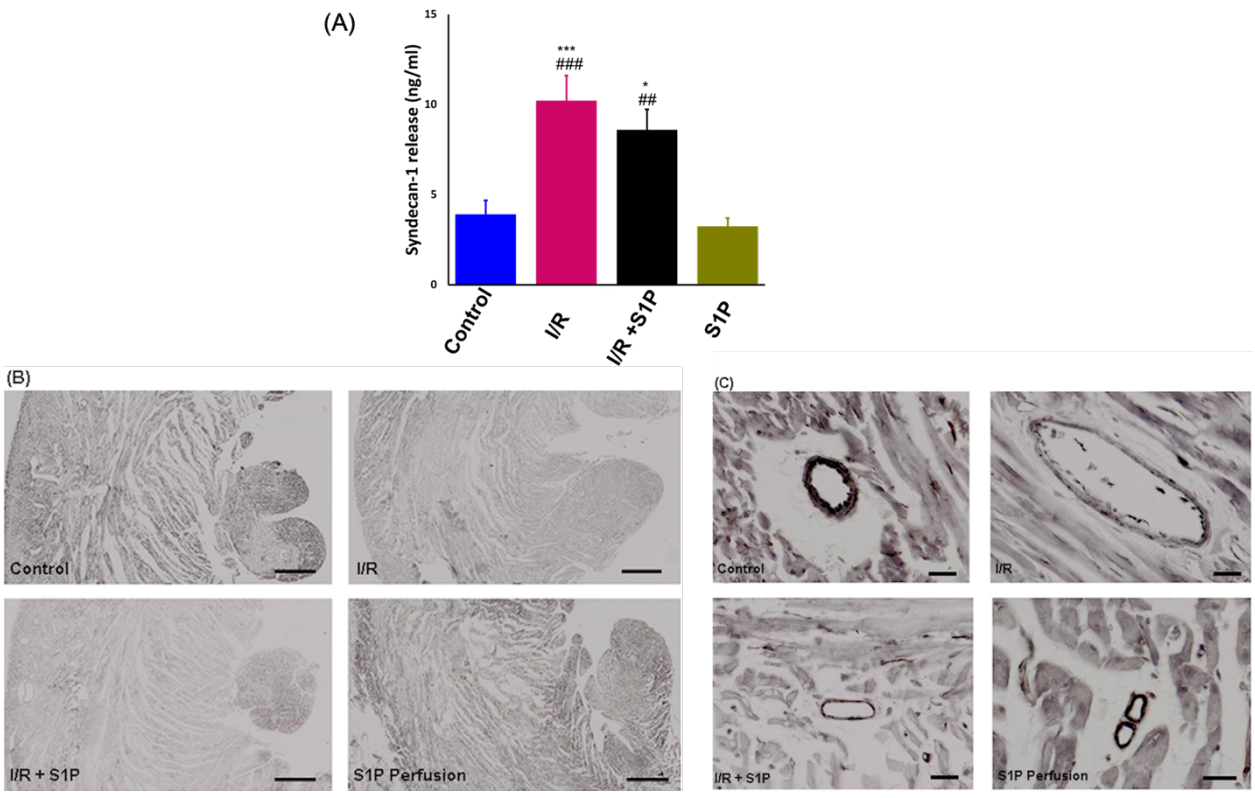

Figure 7: **Syndecan-1 levels in response to ischemia and S1P treatment** A) Quantitative measurement of syndecan-1 release in the coronary effluent of isolated

hearts. *P< 0.05, ***P<0.001 vs. control, ##P< 0.01, ###P<0.001 vs. S1P perfusion group (n≥7 per group). B) Low magnification of chromogenic staining of syndecan-1 in paraffin-embedded sections from the left ventricle of one heart per group. Staining is more intense in non-ischemic hearts (control and S1P) than in ischemic hearts (I/R and I/R+S1P). The slight increase in staining intensity in the regions close to the epicardium and endocardium (arrows) is due to higher numbers of capillaries in the cross-sectional profile of the myocardium in these regions. I/R= Ischemia/reperfusion , S1P= sphingosine-1-phosphate, bar = 500μm C) Representative images of syndecan-1 staining of sections from one left ventricle per group show that Control and S1P perfused hearts have intense staining of larger blood vessels and capillaries, compared to minimal to mild staining observed in the I/R and I/R+S1P groups. Although not all blood vessels types are shown in each of the figures, a similar staining intensity of syndecan-1 was noted in arteries, veins and capillaries within each of the respective groups. (bar = 50μm)

Chromogenic staining of cardiac sections (PFA-fixed/Paraffin-embedded sections) was used for assessing the presence of syndecan-1 in blood vessels. Qualitatively, the staining intensity was markedly greater in non-ischemic groups (control and S1P) compared to that of the ischemic groups (I/R and I/R+S1P) (the outer wall of the left ventricle is shown in Figures 7B and 7C). The regions close to the periphery of the ventricular wall (epicardial and endocardium) show a greater staining intensity than

the central ventricular wall (Figure 7B). However, when observed at higher magnification these regions appear to have a greater density of capillaries per unit area due to the cross-sectional plane of the myocardium at these peripheries and are not related to increased staining intensity of Syndecan-1 compared to larger blood vessels (not shown). No differential staining intensity of Syndecan-1 was observed between arteries, veins and capillaries within each of the groups, i.e. blood vessels were generally of similar staining intensity within each group.

Semiquantitiative analysis based on ordinal data from the scoring system used was not analysed statistically due to the low number of hearts (n=3 per group), however the patterns observed supported the qualitative analysis. The intensity of syndecan-1 immunostaining was decreased in the ischemic group (0.66 ± 0.04) in comparison to the control group (2.56 ± 0.03). Treatment with S1P before ischemia (I/R+S1P) had no effect on syndecan-1 intensity compared to the I/R group (0.56 ± 0.03). Syndecan-1 intensity in hearts treated with S1P without ischemia (S1P) was not majorly different to control hearts but was markedly higher than groups that underwent ischemia with and without pre S1P treatment (2.38 ± 0.04).

Discussion

This study aimed to explore whether the known cardioprotective effect of S1P in isolated rat hearts during I/R injury was mediated via the stabilization of the endothelial glycocalyx. The results showed that S1P treatment of the isolated rat heart subjected to I/R injury led to a significant decrease in infarct size without an effect on shedding of coronary vascular syndecan-1.

In the current study, S1P protected the heart against I/R injury, as was demonstrated by a decrease in infarct size. However, there was no accompanying change in hemodynamic recovery. The effect of S1P on the infarct size in this study was consistent with other studies which used the same dose of S1P [11] indicating that S1P (at a concentration of 10 nM) was effective in reducing the infarction. These results are similar to some other studies that also reported decreases in infarct size without changes in the functional recovery when various drugs were tested. [11,31]

The filtering capacity of the endothelial barrier was determined by measuring heart TWC and the NFR. There was no significant difference in heart TWC between control and ischemic hearts. This finding is consistent with other studies investigating wet-to-dry weight ratio after 15 minutes ischemia and 20 minutes reperfusion[32]. However, the finding that S1P treatment alone led to a significantly decreased TWC is quite interesting. Others have previously shown in the guinea pig that perfusion with Krebs buffer alone leads to increased wet-to-dry weight ratio of the heart [33] and TWC [34] and so our finding may suggest that S1P treatment minimizes any edema caused by perfusion with Krebs buffer. The histological assessment of cardiac edema showed a different picture, wherein

extracellular area was increased by ischemia. The difference in edema between control and I/R reported from the histology data is low (only 6%) and can be explained by the difference in timing of the two measurements; hearts for measurement of TWC were collected after 30 minutes reperfusion whereas hearts for histological analysis were collected after 60 minutes reperfusion. Although not statistically significant our histological results indicated a decreased post -ischemic myocardial edema in S1P pretreated hearts. These results are consistent with those of Straus Straus [35] which indicated that S1P may act to decrease post-ischemic myocardial edema. Possible reasons for the decrease in extracellular edema seen in our study could include S1P induced changes in hemodynamic function but lack of a difference in coronary blood flow between ischemic groups may suggest that hemodynamic changes are not the cause of the decreased extracellular edema in this case.

The increase in the NFR shortly after reperfusion in the ischemic groups compared to the S1P group may indicate an increase in endothelial permeability. However, similar differences in NFR were not seen between the ischemic groups and the control group and may be due to lower NFR in the S1P-treated hearts shortly after reperfusion. It should be noted that the overall protocol seemed to elicit an increase in NFR compared to baseline levels in all groups, however this increase was not significant in S1P treated groups. This, together with the significant decrease in TWC in S1P treated hearts compared to control hearts, may suggest that S1P is eliciting some protection over endothelial permeability. This result is similar to a study that tested the effect of S1P on intact vessels and found that S1P maintains normal vascular permeability by maintaining the endothelial barrier in intact microvessels. [36] Furthermore, another study by Zeng *et*

al[37] found that S1P can stabilize the glycocalyx in cultured rat fat-pad endothelial cells in presence of plasma albumin, and the S1P concentration used in this study was higher than used in the study by Zeng *et al*.

In order to clarify the effect of the S1P on endothelial barrier integrity and cardiac edema, further work may include repeats of histological analysis accompanied by some modification in the methods, such as adding albumin to K-H perfusate to avoid using crystalloid K-H which has low oncotic pressure. In addition, measuring microvascular permeability by using Evan-blue dyed albumin to quantify colloid protein extravasation could be carried out.

The misalignment between the histological results and the heart TWC measurements may be related to the method itself. Some studies have suggested that histological assessment is more specific and more accurate in determination of edema compared with desiccation methods such as measurement of TWC and wet-to-dry-weight ratio. Moreover, it can give adequate preservation of fluid accumulation which allows for distinguishing between extracellular and intracellular edema, a distinction that cannot be made with desiccation methods alone. [34,38,39] However, in this study, only the extracellular edema was measured. The intracellular edema was not evident and thus not measurable, either because the cells were not severely damaged due to the short ischemic duration used in this study, or possibly the intracellular edema resolved after reperfusion as indicated by other studies which showed that intracellular edema is an ischemic event and resolved after reperfusion.[40-43]

The results of this study show that I/R increased the release of syndecan-1 in the coronary effluent, and this may indicate glycocalyx damage. This result was consistent with other studies which also demonstrated that I/R caused damage to the glycocalyx and leads to release of its components into the circulation. [12,44,45] These findings are also consistent with the NFR results described above and may suggest increased endothelial permeability at 5 minutes of reperfusion. In this study, treatment with S1P before ischemia had no effect on syndecan-1 release and suggests that S1P may not protect the heart via stabilization of the glycocalyx. However, it is possible that S1P protects via other components of glycocalyx, such as heparan sulphate and hyaluronic acid. Several other studies testing the protective effect of drugs on the glycocalyx have measured two or more components of glycocalyx [15,26] because some drugs can stabilize one component without any effect on the other components. It is also possible that the timepoint used or the concentration of S1P used in the current study was too low to protect the glycocalyx. In this study, S1P was only administered before ischemia, whereas syndecan-1 shedding occurs during both ischemia and reperfusion [46]. Therefore, administration of S1P at reperfusion may have a different effect on syndecan-1 release. In terms of the concentration of S1P used, Zeng *et al.*[21] found the protective level of S1P in cultured rat fat-pad endothelial cells ranged from 0.2 μM to 1 μM even when there is no protein carrier for S1P, which is above the concentration used in this study. In addition, immuno-staining images of syndecan-1 assessment in coronary vessels supported the ELISA results revealing decreased syndecan-1 after I/R, and similar decreases in syndecan-1 staining in ischemic S1P treated hearts. In any case, the results of this study indicate that S1P, at a concentration that decreased infarct size, did not significantly decrease the elevated

level of syndecan-1 in coronary effluent in ischemic hearts suggesting that this cardioprotective dose of S1P had no effect on the syndecan-1 shedding.

A novel feature of this study was the measurement of syndecan-1 in the cardiac effluent of the isolated heart of rats with global ischemia. Most other studies using isolated perfused hearts to investigate the effects of ischemia reperfusion on the glycocalyx have been carried out using guinea pig hearts. A recent study attempted to assess the role of the glycocalyx in I/R in the mouse heart, however, the levels of syndecan-1 released in response to I/R were too low to measure. [47] The ability to measure syndecan-1 release in response to I/R in the rat heart opens up several possibilities for research on this important structure during I/R. Firstly, it will allow assessment of syndecan-1 shedding during I/R in various well characterized rat disease models. Secondly, it will allow further delineation of the pathways involved in protection of the glycocalyx since species specific antibodies are more widely available for rats than for guinea pigs. Finally, while studies using guinea pig hearts have provided a wealth of information on the ability of certain pharmacological agents to protect the glycocalyx during I/R, the large collateral supply to the heart of guinea pigs means that these studies have focused on global ischemia. Rat hearts have much less collateral circulation [48] and so it is easier to induce regional ischemia in isolated rat hearts. Therefore, the rat model used in our study may open the possibility to test the ability of pharmacological agents to protect the glycocalyx during regional ischemia.

There are some limitations to this study. Firstly, some studies have shown that glycocalyx breakdown is initiated by perfusion with K-H buffer in the absence of albumin[27,49]. In line with this, our study showed some syndecan-1, although minimal,

was found in the coronary effluent of control hearts. However, despite any shedding caused by perfusion with K-H buffer, further shedding was seen as a result of I/R, and S1P treatment was unable to decrease this shedding. We were worried that DMSO treatment in control groups might have caused glycocalyx shedding. However, in preliminary experiments in which syndecan-1 release from hearts perfused with K-H alone were measured, similar values of syndecan-1 were found compared to the control (p=n.s vs. control, vs. S1P perfusion). Secondly, separation of the hearts from the whole animal in the Langendorff perfusion technique removes the hormonal and neural effect on the heart and thus may reduce the clinical relevance of our study. An alternative view here is that this separation of local effects from systemic effects allows for an understanding of the effect of treatments on cardiomyocytes, endothelial cells and fibroblasts alone without the complication of systemic inputs. Furthermore, one might also argue that preconditioning and global ischemia are more relevant to cardiac transplant, a condition where the heart would not be innervated [50]. Thirdly, a heart perfused on the Langendorff system may deteriorate in contractile function at approximately 5–10% per hour [23,24]. Since all hearts were treated the same, one would expect that they would deteriorate at the same rate and therefore any differences are due to the ischemia reperfusion insult and/or the treatment protocols. Finally, despite studies in Langendorff perfused hearts mimicking some of the pathophysiological changes seen in human myocardial infarction[51] , it must be conceded that translation of findings from this model have not been very successful thus far and further work extending these findings to both in vivo and ex vivo models including comorbidities associated with myocardial infarction are needed. [52]

This study showed that I/R leads to increased infarct size, increased release of syndecan-1 in the coronary effluent and histological changes. S1P treatment protects the heart against I/R injury, as was indicated by the decreased infarct size. The fact that S1P pretreatment had no effect on the shedding of syndecan-1, indicates that any such protection is not mediated by maintenance of syndecan-1 in the glycocalyx. It is possible that S1P at this concentration may protect the glycocalyx by stabilizing other glycocalyx components that were not measured in the present study. The study opens up prospects of investigating the role of the glycocalyx in the rat heart, including the more clinically relevant regional I/R model.

Perspectives: This study demonstrated that S1P induced cardioprotection is not mediated by maintenance of syndecan-1 in the endothelial glycocalyx. It also demonstrates for the first time that syndecan-1 levels in the coronary effluent of perfused rat hearts increase in response to ischemia reperfusion injury.

Acknowledgements

We would like to thank the Higher Education Ministry of Libya for sponsoring Hala Araibi's MSc studies. We would also like to thank the National Research Foundation of South Africa (Grant number 85768) who supported Roisin Kelly Laubscher and the South African Medical Research Council (MRC SIR Grant No 29841) who supported Asfree Gwanyanya.

References

1. Nowbar A, Gitto M, Howard J, Francis D, Al-Lamee R. 112 Global and temporal trends in mortality from ischaemic heart disease: statistics from the World Health Organisation. *Heart.* **105**: A93, 2019.

2. Yamazaki S, Fujibayashi Y, Rajagopalan RE, Meerbaum S, Corday E. Effects of staged versus sudden reperfusion after acute coronary occlusion in the dog. *Journal of the American College of Cardiology.* **7**: 564-572, 1986.

3. Dignan RJ, Dyke CM, Abd-Elfattah AS, Lutz HA, Yeh Jr T, Lee KF, Parmar J, Wechsler AS. Coronary artery endothelial cell and smooth muscle dysfunction after global myocardial ischemia. *Ann Thorac Surg.* **53**: 311-317, 1992.

4. van den Berg BM, Vink H, Spaan JA. The endothelial glycocalyx protects against myocardial edema. *Circulation research.* **92**: 592-594, 2003.

5. Tarbell JM, Cancel L. The glycocalyx and its significance in human medicine. *J Intern Med.* **280**: 97-113, 2016.

6. Reitsma S, Slaaf DW, Vink H, Van Zandvoort MA, Oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. *Pfluegers Arch/Eur J Physiol.* **454**: 345-359, 2007.

7. van Haaren PM, VanBavel E, Vink H, Spaan JA. Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy. *Am J Physiol Heart Circ Physiol.* **285**: H2848-H2856, 2003.

8. Patel KD, Nollert MU, McEver RP. P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. *J Cell Biol.* **131**: 1893-1902, 1995.

9. Gouverneur M, van den Berg B, Nieuwdorp M, Stroes E, Vink H. Vasculoprotective properties of the endothelial glycocalyx: effects of fluid shear stress. *Journal of internal medicine.* **259**: 393-400, 2006.

10. Kelly R, Ruane-O'Hora T, Noble M, Drake-Holland A, Snow H. Differential inhibition by hyperglycaemia of shear stress-but not acetylcholine-mediated dilatation in the iliac artery of the anaesthetized pig. *J Physiol (Lond).* **573**: 133-145, 2006.

11. Kelly-Laubscher RF, King JC, Hacking D, Somers S, Hastie S, Stewart T, Imamdin A, Maarman G, Pedretti S, Lecour S. Cardiac preconditioning with sphingosine-1-phosphate requires activation of signal transducer and activator of transcription-3. *Cardiovascular journal of Africa.* **25**: 118, 2014.

12. Mulivor AW, Lipowsky HH. Inflammation-and ischemia-induced shedding of venular glycocalyx. *Am J Physiol Heart Circ Physiol.* **286**: H1672-H1680, 2004.

13. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF α and ischemic preconditioning mediated cardioprotection. *Journal of molecular and cellular cardiology.* **34**: 509-518, 2002.

14. Theilmeier G, Schmidt C, Herrmann J, Keul P, Schäfers M, Herrgott I, Mersmann J, Larmann J, Hermann S, Stypmann J, Schober O, Hildebrand R, Schulz R, Heusch G, Haude M, von Wnuck Lipinski K, Herzog C, Schmitz M, Erbel R, Chun J, Levkau B. High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation.* **114**: 1403-1409, 2006.

15. Zeng Y, Adamson RH, Curry F-RE, Tarbell JM. Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding. *Am J Physiol Heart Circ Physiol.* **306**: H363-H372, 2013.

16. Diebel ME, Diebel LN, Liberati DM. Protective effects of plasma products on the endothelial-glycocalyx barrier following trauma-hemorrhagic shock: is sphingosine-1 phosphate responsible? *Journal of Trauma & Acute Care Surgery.* 2019.

17. Alves NG, Trujillo AN, Breslin JW, Yuan SY. Sphingosine-1-Phosphate reduces hemorrhagic shock and resuscitation-induced microvascular leakage by protecting endothelial mitochondrial integrity. *Shock.* **52**: 423-433, 2019.

18. Zhang L, Zeng M, Fu B. Sphingosine-1-phosphate reduces adhesion of malignant mammary tumor cells MDA-MB-231 to microvessel walls by protecting endothelial surface glycocalyx. *Cell Mol Biol.* **63**: 16-22, 2017.

19. Murata N, Koichi S, Junko K, Tomura H, Yanagita M, Kuwabara A, Michio U, Okajima F. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochemical Journal.* **352**: 809-815, 2000.

20. Adamson RH, Clark JF, Radeva M, Kheirolomoom A, Ferrara K, Curry F. Albumin modulates S1P delivery from red blood cells in perfused microvessels: mechanism of the protein effect. *Am J Physiol Heart Circ Physiol.* **306**: H1011-H1017, 2014.

21. Zeng Y, Liu X-H, Tarbell J, Fu B. Sphingosine 1-phosphate induced synthesis of glycocalyx on endothelial cells. *Experimental cell research.* **339**: 90-95, 2015.

22. Mensah SA, Cheng MJ, Homayoni H, Plouffe BD, Coury AJ, Ebong EE. Regeneration of glycocalyx by heparan sulfate and sphingosine 1-phosphate restores inter-endothelial communication. *PLoS One.* **12**: e0186116, 2017.

23. Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *Journal of molecular and cellular cardiology.* **50**: 940-950, 2011.

24. Skrzypiec-Spring M, Grotthus B, Szeląg A, Schulz R. Isolated heart perfusion according to Langendorff—still viable in the new millennium. *Journal of pharmacological and toxicological methods.* **55**: 113-126, 2007.

25. Annecke T, Chappell D, Chen C, Jacob M, Welsch U, Sommerhoff C, Rehm M, Conzen P, Becker B. Sevoflurane preserves the endothelial glycocalyx against ischaemia–reperfusion injury. *British journal of anaesthesia.* **104**: 414-421, 2010.

26. Chappell D, Jacob M, Hofmann-Kiefer K, Rehm M, Welsch U, Conzen P, Becker BF. Antithrombin reduces shedding of the endothelial glycocalyx following ischaemia/reperfusion. *Cardiovascular research.* **83**: 388-396, 2009.

27. Chen C, Chappell D, Annecke T, Conzen P, Jacob M, Welsch U, Zwissler B, Becker BF. Sevoflurane mitigates shedding of hyaluronan from the coronary endothelium, also during ischemia/reperfusion: an ex vivo animal study. *Hypoxia.* **4**: 81, 2016.

28. Rasband W, ImageJ. US National Institutes of Health, Bethesda, Maryland, USA. **http://rsb.info.nih.gov/ij/**, 1997-2012.

29. Shakkotai PV Mechanistic insights into the acute cardiovascular protection of Atorvastatin, UCL (University College London), 2011.

30. Kliment CR, Oury TD. Extracellular superoxide dismutase protects cardiovascular syndecan-1 from oxidative shedding. *Free Radical Biology and Medicine.* **50**: 1075-1080, 2011.

31. Alizadeh AM, Faghihi M, Sadeghipour HR, MohammadGhasemi F, Khori V. Role of endogenous oxytocin in cardiac ischemic preconditioning. *Regulatory peptides.* **167**: 86-90, 2011.

32. Bruegger D, Rehm M, Jacob M, Chappell D, Stoeckelhuber M, Welsch U, Conzen P, Becker BF. Exogenous nitric oxide requires an endothelial glycocalyx to prevent postischemic coronary vascular leak in guinea pig hearts. *Critical care.* **12**: R73, 2008.

33. Jacob M, Bruegger D, Rehm M, Stoeckelhuber M, Welsch U, Conzen P, Becker BF. The endothelial glycocalyx affords compatibility of Starling's principle and high cardiac interstitial albumin levels. *Cardiovascular research.* **73**: 575-586, 2007.

34. Andrés-Villarreal M, Barba I, Poncelas M, Inserte J, Rodriguez-Palomares J, Pineda V, Garcia-Dorado D. Measuring water distribution in the heart: preventing edema reduces ischemia–reperfusion injury. *Journal of the American Heart Association.* **5**: e003843, 2016.

35. Straus D. Myocardial Edema Attenuation and Sphingosine-1-Phosphate. *Cardiovascular Journal.* **6**: 47-51, 2013.

36. Zhang L, Zeng M, Fan J, Tarbell JM, Curry FRE, Fu BM. Sphingosine-1-phosphate maintains normal vascular permeability by preserving endothelial surface glycocalyx in intact microvessels. *Microcirculation.* **23**: 301-310, 2016.

37. Zeng Y, Adamson RH, Curry F-RE, Tarbell JM. Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding. *American Journal of Physiology-Heart and Circulatory Physiology.* **306**: H363-H372, 2013.

38. Fehrenbach A, Fehrenbach H, Wittwer T, Ochs M, Wahlers T, Richter J. Evaluation of pulmonary edema: stereological versus gravimetrical analysis. *European surgical research.* **33**: 270-278, 2001.

39. Fehrenbach H, Schepelmann D, Albes JM, Bando T, Fischer F, Fehrenbach A, Stolte N, Wahlers T, Richter J. Pulmonary ischemia/reperfusion injury: A quantitative study of structure and function in isolated heart-lungs of the rat. *Anat Rec.* **255**: 84-99, 1999.

40. Askenasy N, Navon G. Continuous monitoring of intracellular volumes in isolated rat hearts during normothermic perfusion and ischemia. *Journal of Magnetic Resonance.* **124**: 42-50, 1997.

41. Askenasy N, Tassini M, Vivi A, Navon G. Intracellular volume measurement and detection of edema: multinuclear NMR studies of intact rat hearts during normothermic ischemia. *Magnetic resonance in medicine.* **33**: 515-520, 1995.

42. Askenasy N, Navon G. Measurements of intracellular volumes by 59Co and 2H/1H NMR and their physiological applications. *NMR Biomed.* **18**: 104-110, 2005.

43. Fernández-Jiménez R, Sánchez-González J, Agüero J, García-Prieto J, López-Martín GJ, García-Ruiz JM, Molina-Iracheta A, Rosselló X, Fernández-Friera L, Pizarro G. Myocardial edema after ischemia/reperfusion is not stable and follows a bimodal pattern: imaging and histological tissue characterization. *Journal of the American College of Cardiology.* **65**: 315-323, 2015.

44. Rehm M, Bruegger D, Christ F, Conzen P, Thiel M, Jacob M, Chappell D, Stoeckelhuber M, Welsch U, Reichart B. CLINICAL PERSPECTIVE. *Circulation.* **116**: 1896-1906, 2007.

45. Chappell D, Heindl B, Jacob M, Annecke T, Chen C, Rehm M, Conzen P, Becker BF. Sevoflurane reduces leukocyte and platelet adhesion after ischemia-reperfusion by protecting the endothelial glycocalyx. *Anesthesiology.* **115**: 483-491, 2011.

46. Annecke T, Fischer J, Hartmann H, Tschoep J, Rehm M, Conzen P, Sommerhoff C, Becker B. Shedding of the coronary endothelial glycocalyx: effects of hypoxia/reoxygenation vs ischaemia/reperfusion. *British journal of anaesthesia.* **107**: 679-686, 2011.

47. Brettner F, Darling J, Baeuml E-C, Mannell H, Frank H-G, Amini M, Hulde N, Kammerer T, Becker BF, Rehm M. Chances and limitations of isolated mouse heart models for investigating the endothelial glycocalyx. *Clinical hemorheology and microcirculation.* **69**: 393-403, 2018.

48. Maxqell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc Res.* **21**: 737-746, 1987.

49. Jacob M, Bruegger D, Rehm M, Welsch U, Conzen P, Becker BF. Contrasting effects of colloid and crystalloid resuscitation fluids on cardiac vascular permeability. *Anesthesiology: The Journal of the American Society of Anesthesiologists.* **104**: 1223-1231, 2006.

50. Grupper A, Gewirtz H, Kushwaha S. Reinnervation post-Heart transplantation. *European heart journal.* **39**: 1799-1806, 2017.

51. Ruiz-Meana M, García-Dorado D. Pathophysiology of ischemia-reperfusion injury: new therapeutic options for acute myocardial infarction. *Revista Española de Cardiología (English Edition).* **62**: 199-209, 2009.

52. Hausenloy DJ, Baxter G, Bell R, Bøtker HE, Davidson SM, Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R. Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. *Basic research in cardiology.* **105**: 677-686, 2010.