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# 3′,4′-Dihydroxyflavonol improves post-ischaemic coronary endothelial function following 7 days reperfusion in sheep

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ABSTRACT

3′,4′-dihydroxyflavonol (DiOHF) is a potent antioxidant that reduces infarct size following myocardial ischaemia–reperfusion. Since oxidative stress induced by myocardial ischaemia–reperfusion impairs endothelium-dependent vasodilatation, we investigated whether DiOHF preserved coronary endothelial function following ischemia–reperfusion. One week after surgery conscious, instrumented sheep were subjected to 1 h of myocardial ischaemia followed by 7 days reperfusion. Immediately before reperfusion, sheep were injected with DiOHF (2 mg/kg iv, $n=4$) or vehicle (dimethyl sulphoxide, $n=4$). Coronary vascular responses to the endothelium-dependent vasodilator acetylcholine (ACh, 0.05–10.0 μg/kg/min iv), sodium nitroprusside and phenylephrine were determined. After ischaemia–reperfusion, $dP/dt_{max}$ decreased from 1511 ± 93 to 1094 ± 53 mmHg/s, $P<0.05$) at 24 h in the vehicle group, but by 7 days had returned towards baseline (1347 ± 91 mm Hg/s). DiOHF prevented the fall in $dP/dt_{max}$. Coronary conductance (CC) was increased (+34 ± 4%) by 10 μg/kg ACh given before ischaemia, but this vasodilatation was significantly reduced after 24 h and 7 days of reperfusion (+7 ± 2%, +15 ± 2%, respectively, both $P<0.05$). DiOHF partially preserved the coronary vasodilator response to ACh after 24 h reperfusion (basal 37 ± 7%, 24 h 18 ± 5%), and after 7 days reperfusion the response had recovered (31 ± 7%). DiOHF significantly decreased infarct size, expressed as a percentage of area-at-risk, by 40% after 7 days reperfusion (vehicle 80 ± 7%, DiOHF 46 ± 11%, $P<0.05$). A single administration of DiOHF, during ischaemia and just prior to reperfusion, reduced infarct size, preserved ventricular contractility and caused a sustained protection against coronary endothelial dysfunction, with all these beneficial actions being preserved for 7 days reperfusion.



## 1. Introduction

Myocardial ischaemia followed by reperfusion (I/R) damages not only myocardial cells but also the coronary vasculature (Sobey and Woodman, 1993). Increased oxidant stress following I/R injury is thought to reduce the availability of endothelium-derived nitric oxide (NO) within the vasculature of the infarct-related coronary arteries (Lefer et al., 1991; Zweier and Talukder, 2006), leading to reduced endothelium-dependent coronary vasodilatation (Ku, 1982; Tsao et al., 1990). The imbalance between superoxide and NO following ischaemia in coronary endothelial cells is believed to activate neutrophils, which plug capillaries and mechanically block myocardial blood flow (Kang and Yang, 2007; Ma et al., 1993). These manifestations of endothelial injury following I/R injury may contribute to progressive flow impairment and tissue hypoperfusion despite complete restoration of epicardial coronary artery patency, which consequently jeopardizes myocardial viability and tissue healing (Kang and Yang, 2007). Thus, the prevention of endothelial dysfunction represents an important therapeutic target to promote recovery from myocardial I/R injury.

Flavonols are polyphenolic metabolites present in commonly consumed fruits, vegetables and beverages which possess a number of beneficial properties, including antioxidant, anti-inflammatory and vasorelaxant activity that is predominantly endothelium-independent (Chan et al., 2000; Flesch et al., 1998; Perez-Vizcaino et al., 2006). Epidemiological studies report an inverse association between dietary flavonol intake and mortality from coronary heart disease (Geleijnse et al., 2002; Lin et al., 2007) and indicate that red wine flavonol-rich polyphenols acutely improve endothelial function in patients with coronary heart disease (Lekakis et al., 2005; Stein et al., 1999). Enhanced vascular NO production (Benito et al., 2002) and increased endothelial cell cGMP content (Flesch et al., 1998) in response to flavonol treatment have been reported. However, an inability of flavonols to change expression and activity of eNOS (Wallerath et al.,

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2005) suggests regulation of NO activity at another level. Endothelial dysfunction occurs very early after reperfusion of the ischaemic myocardium and persists as a long-term injury (Kaeffer et al., 1996; Tsao et al., 1990). Whether flavonols offer short-lived or prolonged protection of the coronary endothelium after I/R injury is unknown.

Like many flavonols, the synthetic flavonol 3′,4′-dihydroxyflavonol (DiOHF) improves vascular function, acting as an antioxidant and a vasorelaxant (Chan et al., 2003, 2000). In experimental studies of I/R injury, we demonstrated DiOHF reduced infarct size in both heart (Wang et al., 2004a, 2009) and brain (Roulston et al., 2008). Moreover, we showed that DiOHF preserved higher levels of NO metabolites in venous outflow from the ischaemic zone, improved coronary blood flow and reduced neutrophil accumulation in the coronary microcirculation within the post-ischaemic myocardium (Wang et al., 2004a). This combination of observations is consistent with preservation of endothelial function. The aim of the present study was to directly assess whether this is the case. Specifically, in conscious sheep, we determined whether DiOHF (i) protected against coronary endothelial injury sustained in the early stages of reperfusion (after 24 h) as well as endothelial dysfunction associated with long-term (7 days) reperfusion and (ii) improved post-ischaemic–reperfusion in microvascular networks, as assessed by regional myocardial blood flow.

## 2. Methods

### 2.1. Animals and study design

This study was performed in eight adult Merino ewes (body weight 31 to 46 kg) housed in air-conditioned rooms, maintained at 22 °C, in individual cages. Large windows allowed a natural light/dark cycle. The sheep were allowed access to water *ad libitum* and were fed lucerne-oaten chaff once daily. The experimental protocol was approved by the Howard Florey Institute Animal Ethics Committee.

### 2.2. Surgical preparation and instrumentation

Sheep were prepared for experimentation in 2 stages, with surgery performed under general anaesthesia induced by intravenous thiopental sodium (15 mg/kg) and, after tracheal intubation, maintained with 1.5–2% isoflourane in air-oxygen (1:1). The left carotid artery was enclosed in a skin loop in the first stage. After 2 weeks recovery, the heart was instrumented via a left thoracotomy in the fourth intercostal space. A solid-state pressure micro-transducer (Konigsberg P6.5, Pasadena, CA, USA) was placed in the left ventricular cavity through a stab wound in the apical dimple to measure left ventricular pressure. The second diagonal branch (D2) of the left anterior descending (LAD) coronary artery was isolated from adjacent tissues, a transit-time flow probe (2 mm, Transonic System Inc, NY, USA) was positioned around it to measure D2 blood flow and a hydraulic vascular cuff (2 mm) was placed around the D2 proximal to the flow probe. To measure regional myocardial contractile function, one pair of ultrasonic crystals (5 MHz, Sonometrics Corporation, Canada) was implanted in the subendocardial layer within the anticipated ischaemic zone of the left ventricle, and another pair was situated in a remote, control area of left ventricle. A silastic cannula filled with heparinised saline (100 U/ml) was inserted into the left atrium for the injection of coloured microspheres. Finally, a transit-time flow probe (20 mm, Transonics Systems Inc, NY, USA) was placed around the ascending aorta to measure aortic flow. All leads and cannulae were tunnelled subcutaneously and exteriorised onto the back between the scapulae. Post-operative analgesia (flunixin meglumine; 1 ml/kg; Mavlab, Qld, Australia) was given at the start of surgery and 2 h post-surgery).

One week after initial instrumentation, a Tygon cannula ('52B', OD 1.7 mm, ID 1.18 mm; Critchley Electrical Products, Sliverwater, N.S.W., Australia) with a side-port extension was inserted into the carotid artery loop of conscious sheep under aseptic conditions to monitor arterial blood pressure. Another catheter was inserted into the jugular vein for intravenous infusion. The patency of the cannulae was maintained by infusing heparinized saline (25 U/ml) at 3.0 ml/h from flush devices (TDF-3WC, Biosensors International, Singapore). To monitor arterial blood pressure (BP) and left atrial pressure (LAP), the corresponding cannulae were connected to pressure transducers (CDXIII, Cobe, Colorado, USA). To measure aortic flow and coronary blood flow (CBF), the respective flow probe leads were connected to a transit-time flow meter (T206, Transonic Systems Inc., Ithaca, New York, USA).

### 2.3. Experimental protocols

With the completion of 24 h of baseline measurements in conscious sheep, ischaemia was initiated by inflating the vascular cuff on the D2 coronary artery. Complete occlusion was maintained for 1 h and was confirmed by a zero D2 flow reading. Vehicle (0.5 ml dimethyl sulphoxide in 4.5 ml polyethylene glycol) or DiOHF (2 mg/kg in vehicle solution) were injected intravenously over the final 5 min of ischaemia. This dose of DiOHF was based on a previous study in conscious sheep in which we demonstrated that DiOHF, at 1, 2 and 5 mg/kg, caused similar degrees of coronary vasodilatation (Wang et al., 2004b). In these conscious sheep, we considered it important to use a low but effective dose of DiOHF to avoid confounding haemodynamic changes that might arise, such as hypotension. Reperfusion commenced with deflation of the occluder. During the first hour of reperfusion, the artery was partly occluded to restrict coronary blood flow to approximately baseline levels. This procedure limited reperfusion hyperaemia, thus reducing the severity of reperfusion arrhythmias (Lucchesi et al., 1976). Lidocaine was given as a bolus injection into the left atria at a dose of 2 mg/kg (Maylab, Queensland, Australia) 10 min before ischaemia and 5 min before reperfusion, and then infused continuously at 1 mg/kg/min (IV) during the first hour of reperfusion to prevent the incidence of ventricular arrhythmias. Buprenorphine (0.3 mg, Reckitt Benckiser, NSW Australia) was injected intravenously after 15 min of ischaemia as an analgesic.

### 2.4. Myocardial function analysis

Digital data of systemic blood pressure, left ventricular pressure (LVP), cardiac output (CO), coronary blood flow (CBF) and segment shortening (SS) were recorded on a computer equipped with a data acquisition program (SonoView 3.1.4, Sonometrics Corporation, Canada). MAP was calculated from the blood pressure recording, and HR, left ventricular end diastolic pressure (LVEDP) and the maximal positive value of the first derivative of left ventricular pressure (LV $dP/dt_{max}$) were analysed from the left ventricular pressure recording. Cardiac output and total peripheral conductance (CO/MAP) were calculated from the aortic flow signal. Mean CBF and mean coronary conductance (CBF/MAP; a measure of coronary vascular tone) were calculated from the coronary flow signals. As an index of regional myocardial contractility, the percentage of myocardial regional segment shortening (SS), defined as [(end diastolic regional length − end systolic regional length)/end diastolic regional length] × 100%, was calculated from the continuous recording of segment length in both regions. Data were collected hourly, except during ischaemia and for the first 2 h of reperfusion when data were collected at 5-min intervals.

### 2.5. Assessment of coronary vascular reactivity

Coronary vascular responses to ACh, SNP and PE were evaluated before ischaemia, and after 24 h and 7 days of reperfusion. The

coronary vascular responses to intravenous infusion of acetylcholine (ACh; 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/kg/min, Sigma Chemical), sodium nitroprusside (SNP; 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 μg/kg/min, Sigma Chemical) and phenylephrine (PE; 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 μg/kg/min, Sigma Chemical) were determined, in this order, in conscious, unstressed animals. Each dose of the vasoactive agents was infused for 5 min before stepping up to the next level. During this experiment, data were collected at 1 min intervals.

### 2.6. Regional myocardial blood flow

Regional myocardial blood flow (RMBF) was measured to determine blood perfusion in different layers of the myocardium. Five measurements of RMBF were taken in each sheep: at baseline, 45 min of ischaemia to validate the absence of collateral circulation and the completeness of ischaemia, and then at 1 h, 24 h and 7 days of reperfusion to monitor the progression of reperfusion flow. At each time point, microspheres ($6\times10^6$ microspheres in 6 ml of 0.05% Tween 80 in saline) labelled with one of five different colours (Dye-Trak VII$^+$, Triton Technology Inc, San Diego, California, USA) were injected into the left atrium. At each time point, reference blood samples (13.5 ml) were drawn from a side-port extension on the carotid artery cannula connected to a withdrawal pump, while microsphere injections were being made (for more details see Supplementary information online). At the end of the experiment, left ventricular tissue sections (~1.0 g), taken from the non-ischaemic zone of the area-at-risk and a remote non-ischaemic region, were cut into 3 pieces (sub-endocardium, mid-myocardium and sub-epicardium). A single tissue block, of similar size to the others, was collected from the centre of the infarcted region. Microspheres were recovered from the tissue blocks and from reference blood samples according to the manufacturer's instructions. Absorbency of the dyes released from the spheres was measured in a spectrophotometer and tissue blood flow rate calculated. (For more details see Supplementary information online).

### 2.7. Analysis of the area-at-risk and infarct size

After the final assessment of coronary vascular reactivity on Day 7, the area of myocardium at risk and infarct size were delineated by double staining as previously reported (Wang et al., 2004a). Briefly, sheep were anaesthetized again and the D2 was re-occluded at the original occlusion site following the reopening of chest chamber through the original incision. Immediately after the heart was arrested by an overdose of pentobarbitone (100 mg/ kg; Virbac, Australia), the ascending aorta was cross-clamped and Evan's blue dye (1.5%, 40 ml, Sigma, Australia) was injected into the atrium to define the myocardium at risk. The heart was rapidly removed and the left ventricle was sectioned perpendicular to its long axis into slices 1 cm in width. All slices were photographed. Infarct area was also photographed after the slices were incubated in 2% triphenyltetrazolium chloride (Sigma Chemicals, USA) at 37 °C for 20 min. All photographs were imported into an image analysis program (MCID-M 2, Imaging Research Inc., Canada), and computerized planimetry was performed. The area-at-risk is expressed as a percentage of the LV, and the infarct size is expressed as a percentage of the area-at-risk.

### 2.8. Statistical analysis

All values are expressed as group mean ± SEM. Difference in infarct size was determined by one-way ANOVA (SigmaStat, version 2.03). All other data were evaluated by one-way ANOVA with repeated measures followed, if appropriate, by Bonferroni's test for multiple comparisons. Differences were considered to be significant when $P<0.05$.

## 3. Results

### 3.1. Hemodynamic parameters

Resting MAP and HR were similar in both groups of conscious sheep (Fig. 1A and B). Myocardial I/R had no significant effect on MAP throughout the experimental period in either group of animals (Fig. 1A). In vehicle-treated sheep HR was significantly ($P<0.05$) elevated at 24 h following reperfusion but not at 7 days, whereas there was no change in HR in DiOHF-treated sheep (Fig. 1B). Resting CBF was similar in both groups of sheep, it decreased to zero flow during ischaemia and returned to basal levels within 24 h in both groups (Fig. 1C).

In vehicle-treated sheep LVEDP was significantly ($P<0.05$) increased from 6.5 ± 1.8 mm Hg to 13.1 ± 3.8 mm Hg at the end of 1 h ischaemia and it remained elevated for the first 12 h (10.4 ± 1.8 mm Hg) of

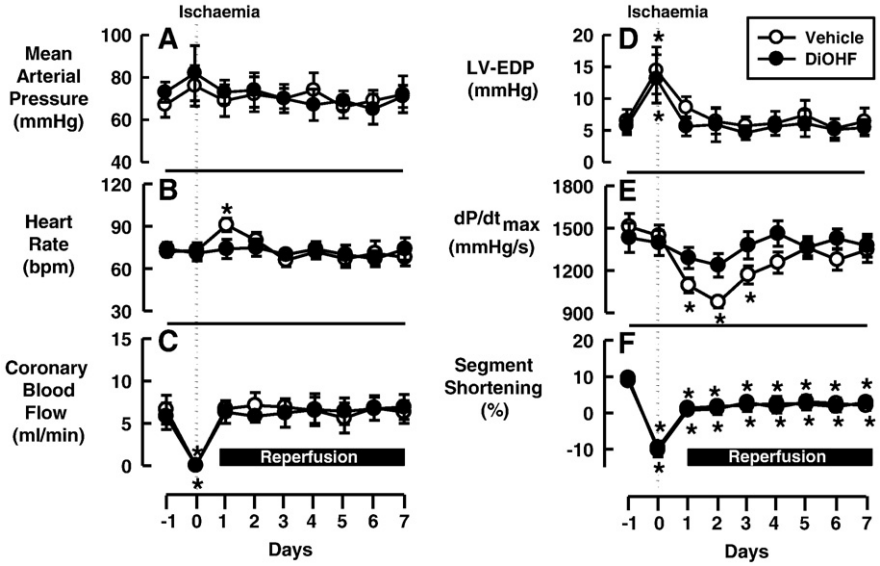


**Fig. 1.** Haemodynamic and coronary effects of DiOHF (2 mg/kg iv bolus) or vehicle during myocardial ischaemia and 7 days reperfusion in conscious sheep ($n=4$ per group). Regional cardiac contractility in the ischaemic zone was measured by segment shortening (%). Global cardiac contractility was measured by changes from baseline of the maximum rate of change in left ventricular pressure ($\Delta dP/dt_{max}$). Data are mean ± sem. *$P<0.05$ *vs* baseline (pre-ischaemia).

reperfusion. In the DiOHF-treated group there was a similar increase in LVEDP during ischaemia. DiOHF significantly reduced the extent of increase in LVEDP at 3 h reperfusion compared to vehicle-treated sheep (8.3 ± 2.8 *vs.* 15.3 ± 2.6 mm Hg, respectively, $P<0.05$, Fig. 1D) and by 12 h ischaemia LVEDP was 6.8 ± 2.3 mm Hg. In both groups, LVEDP returned to basal levels by 24 h reperfusion (vehicle, 8.6 ± 1.7 mm Hg; DiOHF, 5.6 ± 1.5 mm Hg, Fig. 1D). From day 1 to day 3 of reperfusion $dP/dt_{max}$ was markedly reduced in vehicle-treated sheep (basal 1511 ± 93, 24 h 1094 ± 53, 72 h 1169 ± 64 mm Hg/s; $P<0.05$) indicating a prolonged impairment of left ventricular function (Fig. 1E). The administration of DiOHF immediately before reperfusion attenuated the reduction in $dP/dt_{max}$ (basal 1432 ± 5, 24 h 1289 ± 76, 72 h 1378 ± 97 ± mm Hg/s) (Fig. 1E). Regional segment shortening was measured as an index of regional myocardial contractility in the ischaemic zone. Ischaemia induced a significant systolic bulging in both the vehicle and DiOHF-treated groups (Fig. 1F). Although regional systolic function recovered markedly during the early phase of reperfusion, the recovery was incomplete in both groups by the end of the study. There were no changes in regional segment shortening in the non-infarcted zone.

### 3.2. Effects of DiOHF on coronary vascular responses

Acetylcholine (ACh) and sodium nitroprusside caused dose-dependent increases in CBF (not shown) and coronary conductance (CC; Fig. 2) in both groups of conscious sheep before myocardial ischaemia ($P<0.05$). Before ischaemia, CBF increased ~20% and 25% above basal levels with the highest doses of ACh and sodium nitroprusside infusions, respectively. Following 1 h ischaemia and 24 h of reperfusion, coronary vascular responses to the endothelium-dependent vasodilator ACh were significantly ($P<0.05$) reduced in vehicle-treated animals (ΔCC basal 34 ± 4%, 24 h 7 ± 2%), and even after 7 days of reperfusion there was a continued impairment (15 ± 2%, Fig. 2). In the DiOHF group, however, the concentration-dependent coronary vasodilatation in response to ACh was partly preserved after 24 h of reperfusion (ΔCC 18 ± 5% n.s.), and completely recovered (ΔCC 31 ± 7% n.s.) after 7 days of reperfusion (Fig. 2). Coronary responses to the endothelium-independent dilator sodium nitroprusside were unaffected in either vehicle-treated or DiOHF-treated sheep (Fig. 2).

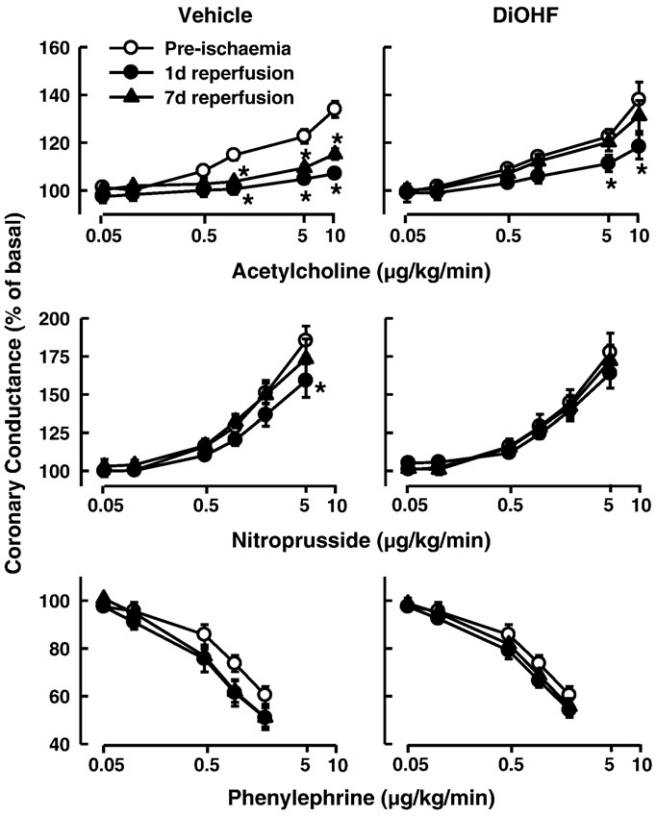


**Fig. 2.** Coronary conductance responses to incremental doses of acetylcholine (0.05–10 μg/kg/min, iv, upper panel), sodium nitroprusside (0.05–5 μg/kg/min, iv, middle panel) and phenylephrine (0.01–2 μg/kg/min, iv, lower panel) in vehicle and DiOHF-treated sheep pre-myocardial ischaemia, and after 1 day and 7 days of reperfusion. Each dose of vasoactive agent was infused for 5 min and the points on the graph are mean ± sem of the final 2 min of infusion. Basal coronary conductance was similar in both groups of sheep before myocardial ischaemia (DiOHF, 0.16 ± 0.03 ml min$^{-1}$ mm Hg$^{-1}$; Vehicle, 0.16 ± 0.02 ml min$^{-1}$ mm Hg$^{-1}$). *$P<0.05$ significant *vs.* pre-ischaemia.

The vasoconstrictor agent phenylephrine caused dose-dependent decreases in CBF (not shown) and coronary conductance (CC; Fig. 2) in both groups of conscious sheep before myocardial ischaemia ($P<0.05$). Prior to ischaemia, CBF decreased ~35% below basal levels over the dose range of phenylephrine infusions (not shown). Myocardial ischemia and reperfusion had no effects on the coronary conductance responses to phenylephrine, and DiOHF treatment did not significantly alter the response to phenylephrine (Fig. 2).

### 3.3. Effects of DiOHF on systemic responses to vasoactive agents

Intravenous infusion of ACh (0.05–10.0 μg/kg/min, IV) had no significant effect on MAP examined before ischaemia or on days 1 and 7 of reperfusion in either vehicle or DiOHF-treated animals (see Supplementary information, Fig. S1). ACh caused concentration-dependent increases of HR, cardiac output and total peripheral conductance that were not influenced by DiOHF treatment and I/R injury (Fig. S2).

Sodium nitroprusside (0.05–5.0 μg/kg/min IV) caused a concentration-dependent fall in MAP that was similar in vehicle and DiOHF-treated animals at baseline, and at days 1 and 7 of reperfusion (see Supplementary information, Fig. S2). Concentration-dependent increases in HR in response to sodium nitroprusside were also comparable between groups throughout the study (Fig. S2). Concentration-dependent increases of CO and total peripheral conductance in response to sodium nitroprusside were significantly reduced at day 1 and day 7 of reperfusion in the vehicle group. In the DiOHF-treated group, the increases in CO and total peripheral conductance in response to sodium nitroprusside were significantly reduced ($P<0.05$) after 24 h reperfusion, but had fully recovered by 7 days reperfusion (Fig. S2).

In both the DiOHF and vehicle-treated groups phenylephrine (0.01–2.0 μg/kg/min, IV) significantly increased MAP in a concentration-dependent manner that was not affected by myocardial I/R (see Supplementary information, Fig. S3). The concentration-dependent reductions of HR, CO and total peripheral conductance in response to phenylephrine were similar in the two groups at the three time points examined (Fig. S3).

### 3.4. Regional myocardial blood flow

Myocardial blood flow in the different layers of LV tissue (epicardium, mid-myocardium, endocardium) within the normal, infarct and area-at-risk zones was comparable in both groups of sheep before ischaemia (Table 1 and Fig. 3). After 1 h ischaemia, RMBF was significantly ($P<0.05$) reduced in the myocardium at risk (including the infarct zone) compared with normally perfused LV tissue in both groups of sheep (Table 1 and Fig. 3). Confirming a previous report in this species (Markovitz et al., 1989), conscious sheep showed very little collateral blood flow (1.0–2.5% of the resting flow rate in the non-necrotic zone within the area-at-risk and less than 0.5% in the infarct zone). RMBF transiently increased to baseline levels in the AR and infarct zones at 1 h reperfusion in both groups (Table 1 and Fig. 3). RMBF in the area-at-risk and infarct zones in vehicle-treated sheep

**Table 1**
Effect of DiOHF (2 mg/kg i.v.) or vehicle treatment on regional myocardial blood flow in *normal* perfused and *infarct zone* left ventricle sheep tissue 7 days after I/R injury.

| Time course | Group | Normal zone | | | Infarct zone |
|---|---|---|---|---|---|
| | | Epi | Mid | Endo | Mid |
| Baseline | Vehicle | 1.17 ± 0.18 | 1.12 ± 0.20 | 1.05 ± 0.24 | 1.04 ± 0.25 |
| | DiOHF | 1.09 ± 0.23 | 1.06 ± 0.23 | 0.96 ± 0.19 | 1.08 ± 0.21 |
| 45′ ischaemia | Vehicle | 1.41 ± 0.36 | 1.25 ± 0.26 | 1.12 ± 0.21 | 0.007 ± 0.002[a] |
| | DiOHF | 1.27 ± 0.21 | 1.19 ± 0.33 | 1.07 ± 0.11 | 0.006 ± 0.005[a] |
| 1 h reperfusion | Vehicle | 1.18 ± 0.29 | 1.11 ± 0.18 | 0.98 ± 0.09 | 1.14 ± 0.28 |
| | DiOHF | 1.21 ± 0.27 | 1.10 ± 0.22 | 0.90 ± 0.07 | 0.97 ± 0.23 |
| 24 h reperfusion | Vehicle | 1.13 ± 0.21 | 1.08 ± 0.18 | 1.01 ± 0.12 | 0.25 ± 0.11[a] |
| | DiOHF | 1.07 ± 0.16 | 1.03 ± 0.24 | 0.99 ± 0.13 | 0.22 ± 0.14[a] |
| 7 days reperfusion | Vehicle | 1.24 ± 0.31 | 1.16 ± 0.24 | 1.11 ± 0.10 | 0.28 ± 0.17[a] |
| | DiOHF | 1.16 ± 0.27 | 1.10 ± 0.19 | 1.03 ± 0.25 | 0.24 ± 0.15[a] |

EPI, MID and ENDO represent epicardium, mid-myocardium and endocardium, respectively. Infarct zone represents the necrotic zone of the myocardium at risk. Data are expressed as milliliters per minute per gram of wet weight and results are mean ± S.E.M., [a]$P<0.05$ *vs.* baseline.

remained significantly ($P<0.05$) lower than baseline levels at days 1 and 7 of reperfusion. DiOHF treatment did not prevent the sustained impairment of RMBF in the infarct zone after 7 days reperfusion, but significantly ($P<0.05$) improved RMBF in all 3 myocardial layers of the AR zone in comparison to the vehicle-treated group (Fig. 3).

### 3.5. Myocardial infarct size

Following 1 h ischaemia and 7 days of reperfusion, the myocardium at risk, expressed as a percentage total left ventricular volume, was comparable in both groups of animals (Fig. 4A). There was a 43% decline in infarct size, expressed as a percentage of the 'at risk' tissue, in DiOHF-treated sheep compared to vehicle-treated sheep ($P<0.05$, Fig. 4B).

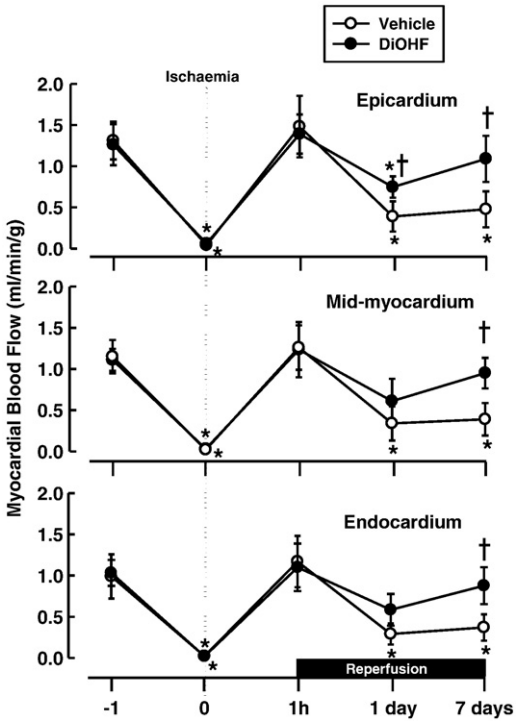


**Fig. 3.** Effects of DiOHF on regional myocardial blood flow in the non-necrotic zone within the area-at-risk. Epicardium (upper panel), mid-myocardium (middle panel), endocardium (lower panel). Data are mean ± sem. *$P<0.05$ vs. baseline, †$P<0.05$ *vs.* vehicle-treated.

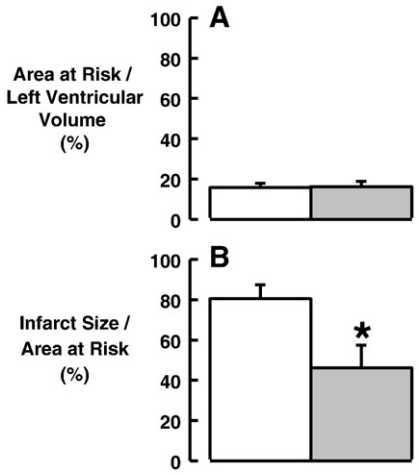


**Fig. 4.** The effect of treatment with DiOHF on the area of myocardium at risk following 1 h ischaemia and on infarct size measured after 1 day and 7 days of reperfusion. A), Area-at-risk is expressed as a percentage of left ventricular (LV) volume. B) Infarct size is expressed as a percentage of area-at-risk. Results are mean ± sem. DiOHF induced a marked limitation of infarct size. *$P<0.05$ *vs.* vehicle-treated animals.

## 4. Discussion

The present study demonstrated that a single administration of the synthetic flavonol DiOHF at the end of 1 h myocardial ischaemia substantially reduced infarct size, protected against reperfusion-induced coronary endothelial dysfunction and improved reperfusion of the non-necrotic zone of the area-at-risk. Importantly, these beneficial effects were sustained over 7 days of reperfusion.

### 4.1. Effects of DiOHF on post-ischaemic endothelial dysfunction

It is well established in many species that coronary vascular responses to ACh are attenuated following myocardial I/R injury (Kaeffer et al., 1996; Sobey et al., 1992; Tsao et al., 1990; Uren et al., 1994; VanBenthuysen et al., 1987). Indeed, myocardial I/R-induced endothelial dysfunction has been detected as early as 2.5 min after reperfusion and has been shown to last from hours to days (Kaeffer et al., 1996; Lefer et al., 1991; Tsao et al., 1990). Similarly, we found an attenuated coronary vascular response in sheep following 1 h ischaemia and early (24 h) reperfusion. Additionally, we showed that the coronary vascular response to ACh remained significantly depressed after 7 days reperfusion in vehicle-treated sheep, confirming that reperfusion-induced endothelial dysfunction is not a transient phenomenon. It is important to note that responses to the endothelium-independent dilator SNP were not significantly affected at any stage following I/R injury, demonstrating that the reduction of coronary artery dilatation was not the result of impaired vascular smooth muscle function, but attributable to impaired endothelial function. Treatment with DiOHF partly preserved the response to Ach after 24 h of reperfusion, and the response had fully returned after 7 days reperfusion, indicating restoration of coronary endothelial function. In contrast to the coronary vasculature, the ACh-induced peripheral vasodilatation, as shown by the increase in total peripheral conductance (Supplementary Material), was not changed by I/R injury or DiOHF treatment. This indicates that the beneficial effect of DiOHF to improve the coronary responsiveness to ACh was not due to an action to increase release of a vasodilator from non-coronary vessels, but was due to a local effect to preserve coronary function.

The demonstrated preservation of coronary endothelial function by DiOHF is likely to contribute to its ability to cause a sustained protection against myocardial reperfusion injury. Evidence supporting this is the finding that acute reperfusion-induced endothelial injury is largely due to damage to endothelial cells, excess superoxide

production, inactivation of NO and an acute inflammatory response characterized by increased adhesion of neutrophils to endothelial cells (Dusting, 1995; Laude et al., 2001; Sobey and Woodman, 1993; Szocs, 2004). Indeed, reperfusion injury to the endothelium may be prevented by free radical scavengers, by prevention of adhesion and/or activation of neutrophils, by exogenous NO supply or by increased endogenous production of NO directly (Dusting, 1995; Laude et al., 2004; Sobey et al., 1992) or via reduction of nitrite to NO (Webb et al., 2004). DiOHF possesses not one, but several, of these beneficial actions. In earlier *in vitro* work we showed that DiOHF, by scavenging superoxide, increased the vasorelaxant activity of ACh in the presence of oxidant stress in the rat aorta (Chan et al., 2003). We also demonstrated that DiOHF reduced superoxide production and significantly increased coronary venous nitrate and nitrite levels *in vivo* in post-ischaemic ovine myocardium (Wang et al., 2004a). Moreover, we have evidence that DiOHF causes selective vasodilatation in the renal and coronary vascular beds, and that this action is NO-dependent (Wang et al., 2004b). DiOHF is a powerful scavenger of superoxide, but also reduces superoxide production by neutrophils by down-regulating components of the NADPH oxidase complex (Jiang et al., 2008). As a result, DiOHF improved epicardial flow and reduced the extent of neutrophil plugging in the coronary microcirculation within the ischaemic zone (Wang et al., 2004a).

Endothelial impairment persists in late reflow, after the burst of superoxide generation that occurs early after reperfusion, suggesting that alterations in endothelial function cannot be explained by simple inactivation of NO by superoxide. Indeed, the loss of endothelial function following ischaemia is paralleled by a loss of eNOS activity (Giraldez et al., 1997), indicating that impaired enzymatic synthesis of NO may also contribute to endothelial dysfunction. In further studies, we found DiOHF treatment shortly before reperfusion maintained the expression of eNOS protein in the post-ischaemic myocardium (Wang et al., unpublished observations). Thus, the action of DiOHF to reverse the imbalance between NO and superoxide and/or neutrophil accumulation may prevent the phenomenon of 'dysfunctional regenerated endothelium', which has been proposed to be responsible for long-term reperfusion-induced endothelial dysfunction (Kaeffer et al., 1996).

### 4.2. Effects of DiOHF on microvascular reflow following ischaemia and prolonged reperfusion

After an initial hyperaemic response at the start of reperfusion, our data indicate progressive RMBF impairment in vehicle-treated sheep within 24 h of reperfusion in both non-necrotic and infarcted zones of myocardium at risk (Fig. 3). After 7 days of reperfusion, RMBF in the risk area remained significantly lower than the levels before ischaemia in these animals, although some improvement could be measured. It is believed that endothelial swelling, cellular edema, cell contracture, and intravascular plugging by neutrophils, platelets and fibrin, contribute to the development of microvascular obstruction during myocardial reperfusion injury (Reffelmann and Kloner, 2002; Rezkalla and Kloner, 2002). Thus, microvascular perfusion may be partly retrieved through the attenuation of these contributors after extended reperfusion. DiOHF appears to target more than one of these mediating factors for microvascular obstruction.

Treatment with DiOHF significantly improved the tissue level of reperfusion flow in the non-necrotic zone of myocardium at risk and prevented the progressive reduction of RMBF that occurred in the vehicle-treated sheep. The "no reflow" phenomenon is observed in ~30% of patients with reperfused anterior wall myocardial ischaemia and is closely associated with decreased contractile function recovery, left ventricular remodelling and a higher incidence of death (Gerber et al., 2000; Ito et al., 1992). The improvement of RMBF by DiOHF would be expected to increase the efficacy and persistence of reperfusion therapy, minimize the level of microvascular damage and thereby protect the jeopardized myocardium.

### 4.3. Effects of DiOHF on myocardial reperfusion injury

In the present study, infarct size, the most important parameter in the determination of lethal myocardial injury, was significantly reduced by 43% after 7 days reperfusion in DiOHF-treated animals. This result is comparable with our earlier studies in anaesthetised sheep, where DiOHF reduced infarct size by 40% after 1 h ischaemia and 2 h reperfusion (Wang et al., 2004a), and with the 43% reduction in infarct size following DiOHF treatment (2 mg/kg iv., daily) in conscious goats following 1 h ischemia and 28 days reperfusion (Wang et al., 2009). These reductions in infarct size are similar to that achieved by ischaemic preconditioning (Wang et al., 2004a). Importantly these findings indicate that the reduction in infarct size following DiOHF is preserved over extended periods of reperfusion.

Treatment with DiOHF prevented the decline in global contractility, measured by $dP/dt_{max}$, but did not significantly improve the recovery of regional myocardial contractility in the post-ischaemia myocardium. This lack of effect on regional contractility may be because the crystals were positioned within the anticipated myocardium at risk, close to the infarcted zone, which could not be expected to recover function. As it was difficult to localise the infarct zone of the non-necrotic zone of the area-at-risk before the necrotic myocardium was clearly identified, it is possible we measured contractility in the infarct, rather than in the surrounding tissue where flow was improved.

In conclusion, a single bolus dose of DiOHF given just prior to reperfusion, reduced infarct size and caused a sustained preservation of endothelial function from early (1 h) until late (7 days) in reperfusion in conscious sheep. In addition, DiOHF attenuated the progressive impairment of regional blood flow to the non-necrotic zone of the myocardium at risk, suggesting a significant reduction in the extent of coronary microvascular obstruction. Importantly, this study demonstrated that the beneficial cardioprotective effects of DiOHF are not transient, but are sustained. These data indicate that DiOHF, with its multiple actions, including antioxidant, anti-inflammatory and eNOS preserving effects, may represent a new adjunctive therapy at the onset of reperfusion, a clinically attractive window for myocardial protection.

## Conflict of interest

O. L. Woodman, G.J Dusting and C.N. May are shareholders in Neuprotect, a company which holds a patent for use of flavonols as a treatment for myocardial ischaemia—reperfusion injury.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.10.001.

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