

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

The Anti-Malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1657421> since 2018-07-16T11:33:59Z

Published version:

DOI:10.1097/SHK.0000000000000963

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches

The Anti-malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction --Manuscript Draft--

Manuscript Number:	SHOCK-D-17-00209R2
Full Title:	The Anti-malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction
Short Title:	Artesunate attenuates cardiac injury
Article Type:	Basic Science Aspects (Animal Subjects)
Keywords:	ischaemia; Myocardial Infarction; pharmacokinetics; heart disease
Corresponding Author:	Christoph Thiernemann, MD, PhD William Harvey Research Institute London, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	William Harvey Research Institute
Corresponding Author's Secondary Institution:	
First Author:	Areeg Khan
First Author Secondary Information:	
Order of Authors:	Areeg Khan
	Amar Kapoor
	Jianmin Chen
	Lukas Martin, M.D.
	Mara Rogazzo
	Thomas Mercier
	Laurent Decosterd
	Massimo Collino
	Christoph Thiernemann
Order of Authors Secondary Information:	
Manuscript Region of Origin:	UNITED KINGDOM
Abstract:	<p>Ischaemic heart disease remains the leading cause of morbidity and mortality in the Western world. Artesunate is the WHO-recommended drug of choice for complicated malaria (with organ failure). The administration of high doses of artesunate is safe in healthy volunteers (up to 8 mg/kg i.v) and patients with severe malaria (2.4 mg/kg i.v). We investigated the effects of artesunate (1 mg/kg) or its active metabolite dihydroartemisinin (DHA; 0.1 mg/kg) in a model of transient myocardial ischaemia/reperfusion (I/R) and evaluated the mechanism of action of the observed cardioprotective effects of artesunate and DHA. We report here for the first time that the administration of artesunate at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of artesunate are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3β, iv) inhibition of NF-κB, and v) activation of the STAT3 (SAFE) pathway. In conclusion, as artesunate has an excellent safety profile, the above data should stimulate clinical trials in patients with acute coronary syndromes.</p>
Response to Reviewers:	Thank you very much for accepting our manuscript, which is very much appreciated.

	Please find attached the manuscript with the suggested final changes. Kind regards, Christoph Thiernemann
--	--

The Anti-malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction

Running title: Khan *et al.*; Artesunate attenuates cardiac injury

Areeg I Khan¹, Amar Kapoor¹, Jianmin Che¹, Lukas Martin¹, Mara Rogazzo², Thomas Mercier³, Laurent Decosterd³, Massimo Collino², Christoph Thiemermann¹

¹Queen Mary University of London, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, UK; ²Department of Drug Science and Technology, University of Turin, Turin, Italy; ³Clinical Pharmacology Laboratory, University Hospital and University of Lausanne, Lausanne, Switzerland.

Address for correspondence and reprints:

Prof Christoph Thiemermann, MD PhD, FBPhS, FRCP, FMedSci, Queen Mary University of London, Barts and The London School of Medicine and Dentistry, William Harvey Research Institute, Centre for Translational Medicine and Therapeutics, Charterhouse Square, London, EC1M 6BQ, UK; E-mail: c.thiemermann@qmul.ac.uk, Tel: + 44 (0) 2078822121, Fax: + 44 (0) 2078828252.

ABSTRACT

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Ischaemic heart disease remains the leading cause of morbidity and mortality in the Western world. Artesunate is the WHO-recommended drug of choice for complicated malaria (with organ failure). The administration of high doses of artesunate is safe in healthy volunteers (up to 8 mg/kg i.v) and patients with severe malaria (2.4 mg/kg i.v). We investigated the effects of artesunate (1 mg/kg) or its active metabolite dihydroartemisinin (DHA; 0.1 mg/kg) in a model of transient myocardial ischaemia/reperfusion (I/R) and evaluated the mechanism of action of the observed cardioprotective effects of artesunate and DHA. We report here for the first time that the administration of artesunate at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of artesunate are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3 β , iv) inhibition of NF- κ B, and v) activation of the STAT3 (SAFE) pathway. In conclusion, as artesunate has an excellent safety profile, the above data should stimulate clinical trials in patients with acute coronary syndromes.

Key words: ischaemia; myocardial infarction; pharmacokinetics; heart disease

INTRODUCTION

Despite major advances in prevention and treatment, ischaemic heart disease remains the leading cause of morbidity in the Western world, and the number of deaths from ischaemic heart disease is also rapidly rising in the developing world. Restoration of early blood flow to the ischaemic myocardium with thrombolysis is presently the most effective therapy to limit infarct size and, thus, preserve cardiac function and geometry following myocardial infarction (MI) (1). Reperfusion alone, however, is inadequate to salvage the damaged myocardium, and may result in 'myocardial ischaemia/reperfusion (I/R) injury'. Various interventions aimed at reducing myocardial I/R injury. As the majority of these interventions are given prior to or during the ischaemic insult in animals, many of them, however, failed to be translated into clinical settings (1). Thus, there continues to be a growing need to discover novel, translatable, safe and cheap interventions that could lessen the global impact of MI on society.

In the past decade, artemisinin and its derivatives have emerged as the most effective drugs for the treatment of severe malaria. Artesunate, the only artemisinin analogue that can be administered intravenously has a very short half-life of approximately 10 min and is rapidly metabolised to dihydroartemisinin (DHA), which has a half-life of approximately 1 h and is regarded as the active metabolite of artesunate (2). In addition to its therapeutic effect in severe malaria (3), artesunate has a multitude of pharmacological properties that include anti-angiogenic as well as anti-inflammatory effects (4, 5). In this regard, the administration of artesunate upon resuscitation protects against the organ injury and dysfunction induced by severe hemorrhage and resuscitation by a mechanism that involves the activation of the Akt-endothelial nitric oxide synthase (eNOS) survival pathway, and the inhibition of glycogen synthase kinase-3 β (GSK-3 β) and nuclear factor kappa B (NF- κ B) (6). The therapeutic effects of artesunate in myocardial I/R injury, however, are unknown.

Here we investigated the effects of artesunate and DHA in a model of transient myocardial I/R in the rat and explored the mechanism of action of the observed cardioprotective effects of artesunate and DHA. Specifically, we have investigated i) the effect of artesunate on the infarct size 2 hours after reperfusion; ii) the mechanisms underlying the observed effects of artesunate including the involvement of the RISK pathway [activation of PI3K signalling pathway (the subsequent phosphorylation Akt on Ser⁴⁷³) and ERK 1/2 signalling pathway] and the SAFE pathway (phosphorylation of STAT3 on Tyr⁷⁰⁵); iii) the involvement of downstream targets of the above pro-survival pathways; phosphorylation of GSK-3 β on Ser⁹, phosphorylation of eNOS on Ser¹¹⁷⁷ and activation NF- κ B (measured as nuclear translocation of p65) and v) the plasma concentration-time profiles of artesunate and DHA in rats.

METHODS

Animal

The experimental protocols using animals were carried out in accordance with the approved guidelines and were approved by the Home Office, London, UK (project license: PPL 70/7348). This study was carried out on 152 male Wistar rats (Charles River Laboratories, Harlow, UK) weighing 240-340 g receiving a standard diet and water *ad libitum*. Rats were randomly assigned to sham, control and treatment groups.

Myocardial Ischaemia/Reperfusion

Myocardial ischaemia/reperfusion was carried out as described previously in this journal (7). Rats were anaesthetised with thiopentone sodium (Intraval, 120 mg/kg i.p. for induction, followed by 10 mg/kg i.v. for maintenance), a tracheostomy was performed and the animals were allowed to artificially respire using a Harvard ventilator (inspiratory oxygen concentration: 30 %; 70 strokes/min tidal volume: 8-10 mL kg⁻¹) throughout the experiment. Body temperature was maintained at 37 ± 1 °C with the aid of a rectal probe thermometer attached to a homeothermic blanket unit (Harvard Apparatus Ltd., Edenbridge, Kent. U.K.). The right carotid artery was cannulated and connected to a pressure transducer (Sensio-Nor 844, Horten, Norway), to monitor mean arterial pressure and heart rate and the right jugular vein was cannulated to administer maintenance anaesthesia and drugs. A parasternal thoracotomy was performed using electrocautery followed by retraction of the thymus and resection of the pericardium. A 6-0 silk suture was placed through the myocardium at the approximate level of the left anterior descending coronary artery. A piece of polythene tubing (Smiths Medical, Watford, UK) flared at one end, was placed over the suture to form a snare occluder. The coronary artery was occluded by tightening the occluder for 25 min, then the occlusion was released to allow reperfusion for 2 h.

Quantification of myocardial tissue injury

At the end of the 2 h reperfusion period, the LAD was re-occluded and 1 ml of Evans Blue dye was administered via the jugular vein to distinguish between perfused and nonperfused (area at risk; AAR) sections of the heart. The heart was excised and immersed in ice-cold 0.9% saline to achieve cardioplegia and sectioned into 3 slices of 3-4 mm to the level of the suture. After right ventricular wall was removed, the non-perfused myocardium was separated from the perfused tissue and was weighed; AAR was expressed by a percentage of the left ventricle. Then the AAR was cut into small pieces and incubated with p-nitroblue tetrazolium (NBT, 0.5 mg/ml) for 30 min at 37 °C. NBT is reduced to a dark blue/purple azole in the presence of reducing compounds, enabling necrotic tissue to be distinguished from viable tissue. The stained tissue was separated from the infarcted tissue, weighed, and the infarct size expressed as a percentage of the AAR.

Western blot analysis

Semi-quantitative western blot analyses in heart tissues (biopsies obtained from the anterior wall – area at risk) were carried out as described previously (8-10). Heart samples were homogenised in 10 % homogenisation buffer and centrifuged at 4,000 RPM for 5 min at 4 °C, after which the supernatant was centrifuged at 14,000 RPM for 40 min to obtain the cytosolic fraction. The pelleted nuclei was resuspended in extraction buffer, incubated in ice for 30 min and centrifuged at 14,000 for 20 min at 4 °C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined on both nuclear and cytosolic extracts using a bicinchoninic acid (BCA) protein assay kit following the manufacturer's directions (Thermo Fisher Scientific, Rockford, IL). Proteins were separated by 8 % sodium dodecyl sulphate-PAGE (SDS-PAGE) and transferred to a polyvinylidenedifluoride membrane, which was then incubated with primary antibody (rabbit anti-total GSK-3 β , dilution 1:200; goat anti-pGSK-3 β Ser⁹ dilution 1:200, rabbit anti-total

1 Akt, dilution 1:1000, mouse anti-pAkt Ser⁴⁷³, dilution 1:1000, goat anti-peNOS Ser¹¹⁷⁷,
2 dilution 1:200, rabbit anti-eNOS, dilution 1:200; rabbit anti-NFκβ p65, dilution 1:1000;
3 rabbit anti-total STAT3, dilution 1:1000, anti-pSTAT3 Tyr⁷⁰⁵, dilution 1:1000) over night at
4 4 °C. Blots were incubated with secondary antibody conjugated with horseradish peroxidase
5 (dilution 1:10,000) for 30 min in at room temperature and developed with the ECL detection
6 system. The immunoreactive bands were visualized by autoradiography and the density of the
7 bands was evaluated densitometrically using the Quantity One 1-D Analysis software (Bio-
8 Rad Laboratories Ltd, Hertfordshire, UK). The membranes were stripped and incubated with
9 β-actin monoclonal antibody for 30 min and subsequently with anti-mouse antibody for 30
10 min, at room temperature, in order to assess gel-loading homogeneity. Relative band intensity
11 was assessed and normalized against parallel β-actin expression. Each group was then
12 adjusted against corresponding sham-control data to establish relative protein expression
13 when compared to sham-control animals.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 32 33 **Experimental Design**

34
35 Animals were either subjected to i) surgical procedure alone with no LAD occlusion or
36 reperfusion, and treated with vehicle (sham), or ii) surgical procedure with LAD occlusion.
37
38 Animals were either treated with vehicle (DMSO 10% v/v or NaHCO₃), artesunate (1 mg/kg
39 in NaHCO₃) or DHA (0.1 mg/kg in DMSO) at the start of reperfusion (bolus injection),
40
41 with/without pre-treatment with inhibitors of PI3K (LY294002) or ERK (U0126) at 10 min
42 before LAD-occlusion (0.3 mg/kg or 0.2 mg/kg, respectively). The dose of artesunate used
43 were based on those that we have recently shown to a) reduce the organ injury/dysfunction
44 and b) activate pro-survival pathways in anaesthetized rats subjected to severe haemorrhage
45 and resuscitation (11). In addition, the dose of 2.4 mg/kg i.v. is the dose used in humans to
46 treat malaria falciparum and, hence, offers the possibility for translational studies. We tried to
47 limit the dose of artesunate used here to the maximum of 2.4 mg/kg, and (in the first instance)
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 tried a dose of 1 mg/kg, which already gave us a significant signal. We are currently
2 evaluating in a single-centre phase II RCT the effects of 2.4 and 4.8 mg/kg i.v. in patients
3 with severe haemorrhage after trauma. The doses of the pathway inhibitors used in this study
4 were selected from the literature (12, 13). As a ‘gold-standard’ reference treatment animals
5 were subjected to surgical procedure followed by either 2 cycles of ischaemic
6 preconditioning (One cycle of IPC: 5 min ischaemia and 5 min reperfusion) with/without pre-
7 treatment with LY294002 or U0126 10 at 10 min before LAD-occlusion.
8
9
10
11
12
13
14
15
16
17
18

19 **LC-MS/MS analysis of artesunate and DHA**

20
21 Blood samples (0.2 ml) were withdrawn from the cannulated carotid artery into heparinized
22 tubes at -5, 5, 15, 30, 45, 60, 120, 180 and 240 min following the i.v. dose (injection of
23 artesunate considered time = 0). The blood samples were centrifuged at 9900 rpm for 3 min
24 to obtain plasma which was stored at -80°C until analysed by liquid chromatography coupled
25 to tandem mass spectrometry (LC-MS/MS). Plasma concentrations of Artesunate (AS) and
26 Dihydroartemisinin (DHA) in samples (about 60µl) were determined by reversed phase LC-
27 MS/MS using an adaptation of the previously described method (14). Rat plasma was
28 purified by protein precipitation with acetonitrile, evaporation, and reconstitution in 10 mM
29 ammonium formate /MeOH (1:1) adjusted to pH 3.9 with formic acid. Separations were done
30 on a 2.1mm x 50mm Atlantis dC18 3 µm analytical column (Waters, Milford, MA, USA).
31 The chromatographic system (CTC Analytics AG, Zwingen, Switzerland) was coupled to a
32 triple stage quadrupole Thermo Quantum Discovery Max mass spectrometer equipped with
33 an electrospray ionization interface (Thermo Fischer Scientific Inc., Waltham, MA). The
34 selected mass transitions were m/z 221.1→163.1 with a collision energy (CE) of 14eV for AS
35 and DHA, and m/z 226.2→168.1 (CE 20eV) for the stable isotope-labelled internal standard
36 dihydroartemisinin-¹³CD₄. Blank rat plasma pool (collected by cardiac puncture of 11 rats)
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

was used for the preparation of matrix-matched calibration and control samples. The calibration curves were fitted by quadratic log-log regression as factor of the peak-area ratio of DHA and artesunate to IS no IS in graphs versus the respective DHA and artesunate concentration in each standard sample. During the analysis, each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Control samples at three concentrations levels (low, medium and high: i.e. 15, 150 and 1500 ng/mL) were assayed throughout rat sample analysis. Inter-assay precision obtained with plasma QCs samples at 15, 150 and 1500 ng/mL of DHA and AS were 12.4, 12.7 and 7.4, and 13.9, 13.2 and 7.5% for DHA and AS, respectively. Mean absolute deviation from nominal values of QCs samples (15, 150 and 1500 ng/mL) during the analysis were 2.2, 5.5, 0.4 %, and 2.1, 0.1, 2.8%, for DHA and AS, respectively. The lower limit of quantification was 10 ng/ml. The Laboratory participates to the External Quality Control program for antimalarial drugs organised by the Worldwide Antimalarial Resistance Network (WWARN).

Statistics

Data is presented as mean \pm standard error of the mean (SEM) of n observations. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The half-lives ($T_{1/2}$) of artesunate and DHA were calculated as $\ln 2/k_e$, considering the slope of the decay of the elimination phase (10-20 and 10-65 min, for artesunate and DHA, respectively). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of artesunate on the infarct size in rats that underwent transient myocardial I/R injury

First, we investigated the effects of a bolus-injection upon reperfusion of artesunate (1 mg/kg; *i.v*) on the infarct size in rats that underwent transient myocardial I/R injury by LAD-occlusion. When compared to sham-animals treated with vehicle, animals subjected to LAD-occlusion and treated with vehicle demonstrated a significant increase in infarct size ($P < 0.0001$, Figure 1). The administration of artesunate, however, significantly reduced the infarct size, compared to vehicle treated animals (or $P < 0.0001$, Figure 1). To determine whether DHA, the active metabolite of many artemisinin derivatives (2), is essential for the observed cardioprotective effects of artesunate, we next investigated the effect of DHA (0.1 mg/kg; *i.v*; at the start of reperfusion) on the infarct size in rats subjected to LAD-occlusion. When compared to rats treated with vehicle alone, the administration of DHA significantly reduced infarct size ($P = 0.01$, Figure 1). To confirm that artesunate is, indeed, metabolised to DHA in rats with myocardial ischaemia and reperfusion, we investigated the plasma concentrations of artesunate and DHA in our experimental setting. Maximal plasma concentrations (C_{\max}) of artesunate and DHA of 39.7 and 223 ng/ml respectively, were detected at a T_{\max} of 10 min in sham animals (Figure 2A). Rats that underwent LAD-occlusion demonstrated a C_{\max} of artesunate and DHA of 67.9 and 187.3 ng/ml respectively, at a T_{\max} of 10 min (Figure 2B). The $T_{1/2}$ of artesunate and DHA were 2 and 3 min, and 13 and 10 min, in sham animals and animals subjected to LAD occlusion, respectively (Figure 2A and 2B).

Effect of artesunate on the phosphorylation of Akt and GSK-3 β , in the hearts of rats that underwent transient myocardial I/R injury

We have previously reported that artesunate activates Akt, inhibits GSK-3 β and inhibits the activation of NF- κ B in liver and kidney of rats subjected to severe haemorrhage (which leads to organ ischaemia) and resuscitation (which leads to reperfusion). In order to elucidate whether the same survival pathways are activated by artesunate in the heart, we have investigated the effects of artesunate on the degree of phosphorylation of Akt on Ser⁴⁷³ and on GSK-3 β on Ser⁹. (15). When compared to sham animals, rats subjected to LAD-occlusion and treated with vehicle demonstrated no changes in the phosphorylation of Akt on Ser⁴⁷³ or GSK-3 β on Ser⁹ (both $P > 0.5$, Figures 3A, 3B). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of both Akt on Ser⁴⁷³ and GSK-3 β on Ser⁹ ($P = 0.005$ and $P = 0.003$, respectively, Figures 3A, 3B), further indicating that the observed cardioprotective effects of artesunate are associated with the activation (phosphorylation) of Akt.

Effect of artesunate on the nuclear translocation of the p65 NF- κ B subunit in the heart of rats that underwent transient myocardial I/R injury

When compared with sham animals, rats subjected to LAD-occlusion exhibited a significant increase in the nuclear translocation of the p65 subunit ($P = 0.008$, Figure 3C) indicating an activation of NF- κ B. The administration of artesunate, however, significantly attenuated the increase in nuclear translocation of p65 and, hence, resulted in an inhibition of the activation of NF- κ B in rats subjected to LAD-occlusion ($P = 0.02$, Figure 3C).

Effect of artesunate on the expression of eNOS in the hearts of rats that underwent transient myocardial I/R injury

As activation of the endothelial nitric oxide synthase (eNOS) has anti-apoptotic, anti-inflammatory, and antioxidant effects, and is hence is cardioprotective (16), we next investigated the effects of artesunate on the degree of phosphorylation of eNOS on Ser¹³³. When compared to sham animals, LAD-occlusion did not affect the phosphorylation of eNOS on Ser¹¹⁷⁷ ($P > 0.5$, Figure 3D). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of eNOS on Ser¹¹⁷⁷ in rats subjected to LAD occlusion and reperfusion ($P = 0.003$, Figure 3D).

Effect of artesunate on the phosphorylation of STAT3, in the heart of rats that underwent transient myocardial I/R injury

As STAT3 is a vital mediator in the SAFE pathway and is known to mediate cardioprotection when activated by mechanical procedures (e.g. pre- and postconditioning) (17) (18), we next investigated the effects of artesunate on the degree of phosphorylation of STAT3 on Tyr⁷⁰⁵. When compared to sham animals, rats subjected to LAD-occlusion demonstrated no change in the phosphorylation of STAT3 on Tyr⁷⁰⁵ ($P > 0.05$, Figures 3E). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of STAT3 on Tyr⁷⁰⁵ ($P = 0.03$, Figures 3E).

Effect of artesunate on the infarct size, in animals that underwent transient myocardial I/R injury following inhibition of survival pathways

The above data provide some evidence that artesunate activates key elements of the RISK pathways after LAD-occlusion and reperfusion. It is not clear from the above data whether inhibition of key elements of the RISK pathway also attenuates the cardioprotective effects of artesunate (cause-effect relationship). Thus, we investigated the roles of PI3K/Akt and ERK 1/2 (e.g. the signalling events know to activate the RISK survival pathway (12, 13) in the cardioprotective effects of artesunate. Therefore, rats were pre-treated (10 min prior to

1 ischaemia) with inhibitors of either PI3K (LY294002, 0.3 mg/kg; *i.v*) (12) or ERK 1/2
2 (U0126, 0.2 mg/kg; *i.v*) (13). When compared to LAD-subjected animals treated with
3 artesunate and no prior inhibition of either PI3K or ERK, the administration of either
4 LY294002 or U0126 significantly abolished the reduction of the infarct size by artesunate,
5 indicating that the activation by artesunate of both the PI3K/Akt and the ERK 1/2 pathways
6 are essential for the cardioprotective effects of artesunate ($P < 0.0001$, Figure 4).
7
8
9
10
11
12
13
14
15
16

17 **Effect of inhibition of the RISK pathway on infarct size, in rats that underwent** 18 **transient myocardial I/R injury subsequent to ischaemic preconditioning (IPC)** 19

20 Having shown that activation by artesunate of both the PI3K/Akt and the ERK 1/2 pathways
21 are essential for the cardioprotective effects of artesunate, we speculated that artesunate
22 protects the heart through mechanisms that are similar to those of ischaemic preconditioning
23 (IPC). In order to confirm that activation of the RISK pathway is pivotal in mediating the
24 IPC-induced cardioprotection, rats were treated with LY294002 or U0126 at 10 min prior to
25 initiating the IPC stimulus. When compared to animals subjected to LAD-occlusion and
26 treated with vehicle, 2 cycles of IPC before occlusion of the LAD resulted in a significant
27 reduction in infarct size ($P < 0.0001$, Figure 5). Like the cardioprotective effect of artesunate,
28 the cardioprotective effect afforded by IPC was also abolished by LY294002 and U0126 ($P =$
29 0.0004 and $P = 0.002$, respectively, Figure 5), indicating that the activation of both the
30 PI3K/Akt and the ERK 1/2 pathways are essential for the cardioprotective effects of both
31 artesunate and IPC.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

DISCUSSION

We report here for the first time that a single *i.v.* injection upon reperfusion of the safe, antimalarial drug artesunate reduces myocardial infarct size in the rat *in vivo*. Notably, the observed beneficial effects of artesunate were associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3 β , iv) inhibition of NF- κ B, and v) activation of the STAT3 (SAFE) pathway, while inhibitors of the RISK pathway (Both PI3K and ERK) abolished the cardioprotective effects of artesunate.

Activation of the PI3K/Akt/eNOS survival pathway is known to protect the myocardium against I/R injury (19). Indeed, we report here that the cardioprotective effects afforded by artesunate were a) associated with phosphorylation of Akt on Ser⁴⁷³, and b) abolished by the specific PI3K-inhibitor LY294002, suggesting that the artesunate-induced cardioprotection is secondary to the activation of the PI3K/Akt-survival pathway. Akt phosphorylates eNOS and the resulting eNOS activation has anti-apoptotic, anti-inflammatory and antioxidant effects. The administration of artesunate was associated with an increase in the phosphorylation of eNOS on Ser¹¹⁷⁷, secondary to activation of the PI3K/Akt pathway. Moreover, we report here that the cardioprotective effects of artesunate were associated with Ser⁹ phosphorylation and, hence, inactivation of GSK-3 β in the heart, both of which are known to modulate various cellular processes including survival and apoptosis. Moreover, inhibition of GSK-3 β prevents the nuclear translocation of p65 and, hence the activation of NF- κ B (20). The observed cardioprotective effects of artesunate were associated with both Ser⁹ phosphorylation of GSK-3 β (see above) and reduction of the nuclear translocation of p65 from the cytosol to the nucleus (in the ischaemic heart). Recently, Collino and colleagues demonstrated that inhibition of GSK-3 β with either insulin or TDZD-8 was associated with a significant reduction of the nuclear NF- κ B activity, in diabetic rats subjected to cerebral I/R injury (21).

1 Blockade of NF- κ B activation by NF- κ B decoys (22), or a vitamin E-like antioxidant (23) has
2 been reported to attenuate myocardial I/R injury and to improve functional recovery of the
3 heart after an ischaemic event. Thus, it is likely that the observed effects of artesunate are (at
4 least in part) attributable to the reduced activation of NF- κ B.
5
6
7
8
9

10
11 Members of the MAPK cascade have been implicated in regulating myocyte survival after
12 I/R injury and oxidative stress. ERK 1/2 signalling has been identified as one of the major
13 components of the RISK pathway. We report here that the ERK 1/2 inhibitor, U0126,
14 abolished the cardioprotective effects afforded by artesunate, indicating that the activation of
15 ERK 1/2 importantly contributes to the cardioprotective effects of artesunate. Indeed, the
16 activation of the ERK pathway during reperfusion leads to cardioprotection (24, 25).
17
18
19
20
21
22
23
24
25
26
27

28
29 Additionally, activation of the SAFE pathway may mediate the cardioprotective effects of
30 ischaemic pre- and postconditioning. STAT3 is a latent transcription factor, plays a role in
31 various cellular functions including proliferation, differentiation and cell survival (26). We
32 demonstrate here that the cardioprotective effects of artesunate in the rat heart were also
33 associated with activation of STAT3. There is good evidence that activation of STAT3 in the
34 heart confers cardioprotection as the myocardial infarct size caused by I/R in mice with
35 cardiac-specific expression of constitutively active STAT3 is significantly smaller than the
36 infarct size caused by I/R results in non-transgenic littermates (27). Our results clearly show
37 that artesunate caused activation of both the RISK and the SAFE pathways. Thus, our study
38 confirm previous data which have identified a role for SAFE signaling in the initiation of the
39 activation of the RISK pathway, which plays a pivotal role in the cardioprotection afforded
40 by pre- and post-conditioning (28, 29). Whether enhanced activation of SAFE further
41 promotes RISK activation or vice versa cannot be distinguished in our study. However, our
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 data suggest that targeting a single pathway as a sole therapeutic target may not be sufficient
2 (or at least less effective) than targeting both pathways. Interestingly, we also report that
3 artesunate causes both STAT3 activation and NF- κ B inhibition. Inhibition of NF- κ B
4 activation secondary to STAT3 activation inhibits NF- κ B-mediated hypertrophic and
5 inflammatory gene expression by blocking NF- κ B activation in the heart (30). However,
6 exactly how this occurs and the temporal pattern of the reported cross-talk between STAT3
7 NF- κ B are still unclear.
8
9
10
11
12
13
14
15
16
17
18

19 In vivo, artesunate is rapidly hydrolysed to DHA, which is the common active metabolite that
20 mediates the beneficial effects of many artemisinin derivatives in patients with malaria (31).
21 The conversion from artesunate to DHA occurs rapidly following *i.v.* administration,
22 indicated by the swift decline in artesunate concentrations in the early post-dose period (32).
23 DHA metabolism occurs through conjugation by the UDP-glucuronosyltransferase system,
24 with UGT1A9 and UGT2B7 being the main responsible isoforms (33). The metabolism of
25 DHA occurs more slowly, with an average half-life of DHA of 1 – 2 h after bolus injection of
26 artesunate (32). In our study, peak plasma concentrations of both artesunate (39.7 ng/ml) and
27 DHA (223 ng/ml) were achieved in the rat within 10 min of the injection of artesunate. This
28 demonstrates a very rapid conversion of artesunate to DHA in the rat, and this metabolism
29 was unaltered in rats subjected to myocardial I/R. The peak plasma concentrations seen here
30 in the sham treated rats are much lower than those exhibited in healthy human volunteers,
31 where a similar dose of artesunate (1 mg/kg) resulted in a peak plasma concentration of
32 artesunate and DHA of around 6128 ng/ml and 800 ng/ml, respectively (34). The efficacy of
33 artesunate in malaria has been attributed to i) attaining a high rapid initial C_{max} , and ii) its
34 swift hydrolysis to DHA (35). This could mean that DHA is more potent than artesunate in
35 reducing parasite load in malaria, but also indicates that DHA may mediate the
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

cardioprotective effects of artesunate. We demonstrate here that a dose of 0.1 mg/kg of DHA caused a significant reduction in infarct size, while the same dose of artesunate was not effective (data not shown). This indicates that DHA is the effective mediator of the cardioprotective effects of artesunate (although we cannot exclude that artesunate has at least some cardioprotective effects in its own right).

Limitations of the study: We report that the i.v. injection of artesunate upon reperfusion reduces infarct size when measured in anaesthetized, ventilated rats after a relatively short (2 hours) period of reperfusion. Further studies are warranted that confirm that artesunate reduces the size of necrosis and improves contractility (function) after longer periods of reperfusion (ideally 1-2 weeks). We are unable to provide definite prove that DHA is, indeed, the active metabolite, which mediates the cardioprotective effects of artesunate, as we are unable to block the metabolism of artesunate to DHA. We cannot exclude that the pathway inhibitors used in this study have ‘unknown’, non-specific effects, which may have contributed to ‘prevention’ of the cardioprotective effects of artesunate documented in this study. This could, of course, be true for any pharmacological intervention used during an in vivo experiment and, hence, is not a specific limitation of this study.

In conclusion, we report here for the first time that the administration of low doses (1 mg/kg i.v) artesunate at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of artesunate are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3 β , iv) inhibition of NF- κ B, and v) activation of the STAT3 (SAFE) pathway. Most notably, the cardioprotective effects of artesunate were abolished by inhibition of PI3K and ERK1/2 indicating that activation by artesunate of these pathways is pivotal for the cardioprotective

1 effects of artesunate. Artesunate is rapidly metabolised to DHA *in vivo* and this metabolism is
2 not altered in animals with myocardial infarction. DHA also reduces myocardial infarct size
3 in the rat indicating that DHA may be the active metabolite of artesunate. As the
4 administration of higher doses of artesunate is safe in healthy volunteers and patients with
5 severe malaria, artesunate may represent a novel highly translatable approach for the therapy
6 of acute MI in man.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

REFERENCES

1. Bolli R, Becker L, Gross G, Mentzer R, Jr., Balshaw D, Lathrop DA, Ischemia NWGotToTfPtHf: Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res* 95:125-134, 2004.
2. Krishna S, Uhlemann AC, Haynes RK: Artemisinins: mechanisms of action and potential for resistance. *Drug Resist Updat* 7:233-244, 2004.
3. Haynes RK: Artemisinin and derivatives: the future for malaria treatment? *Curr Opin Infect Dis* 14:719-726, 2001.
4. Aldieri E, Atragene D, Bergandi L, Riganti C, Costamagna C, Bosia A, Ghigo D: Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-kB activation. *FEBS Lett* 552:141-144, 2003.
5. Wartenberg M, Wolf S, Budde P, Grunheck F, Acker H, Hescheler J, Wartenberg G, Sauer H: The antimalaria agent artemisinin exerts antiangiogenic effects in mouse embryonic stem cell-derived embryoid bodies. *Lab Invest* 83:1647-1655, 2003.
6. Sordi R, Nandra KK, Chiazza F, Johnson FL, Cabrera CP, Torrance HD, Yamada N, Patel NS, Barnes MR, Brohi K, et al.: Artesunate Protects Against the Organ Injury and Dysfunction Induced by Severe Hemorrhage and Resuscitation. *Ann Surg* 265:408-417, 2017.
7. Sivarajah A, McDonald MC, Thiernemann C: The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. *Shock* 26:154-161, 2006.
8. Chen J, Kieswich JE, Chiazza F, Moyes AJ, Gobbetti T, Purvis GS, Salvatori DC, Patel NS, Perretti M, Hobbs AJ, et al.: IkappaB Kinase Inhibitor Attenuates Sepsis-Induced Cardiac Dysfunction in CKD. *J Am Soc Nephrol* 28:94-105, 2017.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
9. Martin L, Horst K, Chiazza F, Oggero S, Collino M, Brandenburg K, Hildebrand F, Marx G, Thiemermann C, Schuerholz T: The synthetic antimicrobial peptide 19-2.5 attenuates septic cardiomyopathy and prevents down-regulation of SERCA2 in polymicrobial sepsis. *Sci Rep* 6:37277, 2016.
10. Yamada N, Martin LB, Zechendorf E, Purvis GS, Chiazza F, Varrone B, Collino M, Shepherd J, Heinbockel L, Gutschmann T, et al.: Novel Synthetic, Host-defense Peptide Protects Against Organ Injury/Dysfunction in a Rat Model of Severe Hemorrhagic Shock. *Ann Surg* 2017.
11. Sordi R, Nandra KK, Chiazza F, Johnson FL, Cabrera CP, Torrance HD, Yamada N, Patel NS, Barnes MR, Brohi K, et al.: Artesunate Protects Against the Organ Injury and Dysfunction Induced by Severe Hemorrhage and Resuscitation. *Ann Surg* 2016.
12. Wang Y, Zhang ZZ, Wu Y, Ke JJ, He XH, Wang YL: Quercetin postconditioning attenuates myocardial ischemia/reperfusion injury in rats through the PI3K/Akt pathway. *Braz J Med Biol Res* 46:861-867, 2013.
13. Ye Y, Zhao Z, Xu H, Zhang X, Su X, Yang Y, Yu X, He X: Activation of Sphingosine 1-Phosphate Receptor 1 Enhances Hippocampus Neurogenesis in a Rat Model of Traumatic Brain Injury: An Involvement of MEK/Erk Signaling Pathway. *Neural Plast* 2016:8072156, 2016.
14. Hodel EM, Zanolari B, Mercier T, Biollaz J, Keiser J, Oliaro P, Genton B, Decosterd LA: A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* 877:867-886, 2009.
15. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785-789, 1995.

16. Forstermann U, Sessa WC: Nitric oxide synthases: regulation and function. *Eur Heart J* 33:829-837, 837a-837d, 2012.
17. Lacerda L, Somers S, Opie LH, Lecour S: Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovasc Res* 84:201-208, 2009.
18. Frias MA, Pedretti S, Hacking D, Somers S, Lacerda L, Opie LH, James RW, Lecour S: HDL protects against ischemia reperfusion injury by preserving mitochondrial integrity. *Atherosclerosis* 228:110-116, 2013.
19. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K: Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 101:660-667, 2000.
20. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR: Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406:86-90, 2000.
21. Collino M, Aragno M, Castiglia S, Tomasinelli C, Thiemermann C, Boccuzzi G, Fantozzi R: Insulin reduces cerebral ischemia/reperfusion injury in the hippocampus of diabetic rats: a role for glycogen synthase kinase-3beta. *Diabetes* 58:235-242, 2009.
22. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki J, et al.: In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med* 3:894-899, 1997.
23. Altavilla D, Deodato B, Campo GM, Arlotta M, Miano M, Squadrito G, Saitta A, Cucinotta D, Ceccarelli S, Ferlito M, et al.: IRFI 042, a novel dual vitamin E-like antioxidant, inhibits activation of nuclear factor-kappaB and reduces the inflammatory response in myocardial ischemia-reperfusion injury. *Cardiovasc Res* 47:515-528, 2000.

24. Baxter GF, Mocanu MM, Brar BK, Latchman DS, Yellon DM: Cardioprotective effects of transforming growth factor-beta1 during early reoxygenation or reperfusion are mediated by p42/p44 MAPK. *J Cardiovasc Pharmacol* 38:930-939, 2001.
25. Schulman D, Latchman DS, Yellon DM: Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol* 283:H1481-1488, 2002.
26. Bromberg J, Darnell JE, Jr.: The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19:2468-2473, 2000.
27. Oshima Y, Fujio Y, Nakanishi T, Itoh N, Yamamoto Y, Negoro S, Tanaka K, Kishimoto T, Kawase I, Azuma J: STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart. *Cardiovasc Res* 65:428-435, 2005.
28. Drenger B, Ostrovsky IA, Barak M, Nechemia-Arbely Y, Ziv E, Axelrod JH: Diabetes blockade of sevoflurane postconditioning is not restored by insulin in the rat heart: phosphorylated signal transducer and activator of transcription 3- and phosphatidylinositol 3-kinase-mediated inhibition. *Anesthesiology* 114:1364-1372, 2011.
29. Goodman MD, Koch SE, Fuller-Bicer GA, Butler KL: Regulating RISK: a role for JAK-STAT signaling in postconditioning? *Am J Physiol Heart Circ Physiol* 295:H1649-1656, 2008.
30. Verma SK, Krishnamurthy P, Barefield D, Singh N, Gupta R, Lambers E, Thal M, Mackie A, Hoxha E, Ramirez V, et al.: Interleukin-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-kappaB. *Circulation* 126:418-429, 2012.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
31. White NJ: Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives. *Trans R Soc Trop Med Hyg* 88 Suppl 1:S41-43, 1994.
32. Morris CA, Duparc S, Borghini-Fuhrer I, Jung D, Shin CS, Fleckenstein L: Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration. *Malar J* 10:263, 2011.
33. Ilett KF, Ethell BT, Maggs JL, Davis TM, Batty KT, Burchell B, Binh TQ, Thu le TA, Hung NC, Pirmohamed M, et al.: Glucuronidation of dihydroartemisinin in vivo and by human liver microsomes and expressed UDP-glucuronosyltransferases. *Drug Metab Dispos* 30:1005-1012, 2002.
34. Li Q, Cantilena LR, Leary KJ, Saviolakis GA, Miller RS, Melendez V, Weina PJ: Pharmacokinetic profiles of artesunate after single intravenous doses at 0.5, 1, 2, 4, and 8 mg/kg in healthy volunteers: a phase I study. *Am J Trop Med Hyg* 81:615-621, 2009.
35. Batty KT, Le AT, Ilett KF, Nguyen PT, Powell SM, Nguyen CH, Truong XM, Vuong VC, Huynh VT, Tran QB, et al.: A pharmacokinetic and pharmacodynamic study of artesunate for vivax malaria. *Am J Trop Med Hyg* 59:823-827, 1998.

AUTHOR CONTRIBUTIONS

CT and AIK designed research; AIK, AK, JC, MR, TM, LD and MC performed research; AIK, AK, JC, LM, MR, TM, LD, and MC analysed data; and AIK, JC, LM and CT wrote the paper.

CONFLICTS OF INTEREST AND FUNDING SOURCES

AIK is supported by a PhD-studentship of the Medical Research Council. AK is supported in part by the William Harvey Research Foundation and by the British Heart foundation (PG/11/30/28849). JC is funded by China Scholarship Council. LM is funded by the Deutsche Forschungsgemeinschaft (DFG, MA 7082/1–1). Queen Mary University of London holds IP for the use of artesunate in myocardial infarction and other conditions associated with ischaemia-reperfusion injury (United States Application No: 14/125032). CT is a named inventor on the above patents.

FIGURES LEGENDS

Figure 1. Effect of artesunate or dihydroartemisinin on infarct size in rats that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham, $n = 6$), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle ($n = 10$), artesunate (ARS; 1 mg/kg; $n = 10$) or dihydroartemisinin (DHA; 0.1 mg/kg, $n = 6$) at the start of reperfusion. AAR, area at risk. Data are expressed as means \pm SEM for n number of observations. $*P < 0.0001$ vs. MI + vehicle; $^{\S}P < 0.0001$ vs. MI + ARS.

Figure 2. Plasma concentration-time profiles of artesunate and dihydroartemisinin following a single intravenous dose of artesunate. Rats were either subjected to (left) surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD) or (right) surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion. Rats were treated with artesunate (ARS; 1 mg/kg) at the start of reperfusion. Plasma concentrations of artesunate and dihydroartemisinin (DHA) were measured in blood samples withdrawn from the cannulated carotid artery at -5, 5, 15, 30, 45, 60, 120, 180 and 240 min following bolus-injection of artesunate. Data are expressed as means \pm SEM for four numbers of observations.

Figure 3. Effect of artesunate on signalling pathways in the heart of rats that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle or artesunate (ARS; 1 mg/kg) at the start of

reperfusion. Heart samples were collected at the end of the 120 min reperfusion period. Densitometric analysis of the bands is expressed as relative optical density (O.D) of (A) Akt phosphorylation (pSer⁴⁷³) corrected for the corresponding total Akt content; (B) glycogen synthase kinase (GSK)-3 β phosphorylation (pSer⁹) corrected for the corresponding total GSK-3 β content; (C) NF- κ B p65 subunit levels in both cytosol and nuclear fractions expressed as a nucleus/cytosol ratio; (D) endothelial nitric oxide synthase (eNOS) phosphorylation (pSer¹¹⁷⁷) corrected for the corresponding total eNOS content; (E) signal transducer and activator of transcription 3 (STAT3) phosphorylation at Tyr⁷⁰⁵ (pSTAT3⁷⁰⁵), corrected for the corresponding total STAT3 content. All values were normalized using the related sham-operated band. Data is expressed as means \pm SEM for four observations. * $P < 0.0001$ vs. *MI + vehicle*.

Figure 4. Effect of inhibition of components of the RISK pathway on infarct size in rats treated with artesunate that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham, $n = 6$), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle ($n = 10$) or artesunate (ARS; 1 mg/kg; $n = 10$) at the start of reperfusion with or without pre-treatment with LY29400 (LY; 0.3 mg/kg; $n = 10$) or U0126 (0.2 mg/kg; $n = 10$) at 10 min before LAD-occlusion. AAR, area at risk. Data are expressed as means \pm SEM for n number of observations. * $P < 0.0001$ vs. *MI + vehicle*; ^s $P < 0.0001$ vs. *MI + ARS*.

Figure 5. Effect of inhibition of components of the RISK pathway on infarct size in rats that underwent transient myocardial I/R injury subsequent to an IPC stimulus. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior

1 descending coronary artery (LAD), and treated with vehicle (sham, $n = 6$), or surgical
2 procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion. For ischaemic
3 preconditioning, 2 cycles of ischaemic preconditioning (IPC; 1 cycle: 5 min ischaemia and 5
4 min reperfusion) have been performed with or without pre-treatment with LY29400 (LY; 0.3
5 mg/kg; $n = 6$) or U0126 (0.2 mg/kg; $n = 6$) at 10 min before LAD-occlusion. AAR, area at
6 risk. Data are expressed as means \pm SEM for n number of observations. $*P < 0.0001$ vs. *MI +*
7 *vehicle*; $^{\S}P < 0.0001$ vs. *MI + IPC + Vehicle*.
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 1

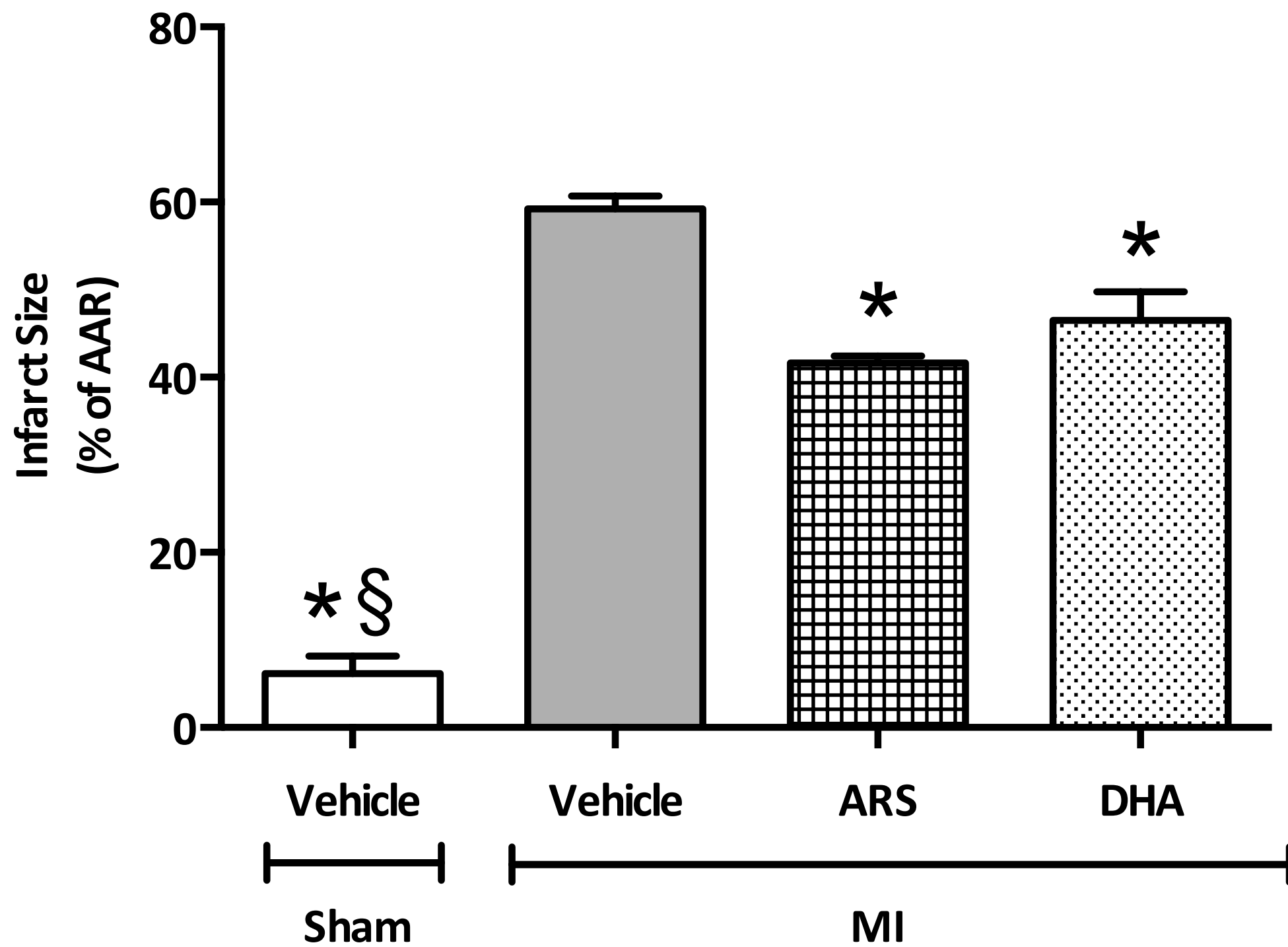
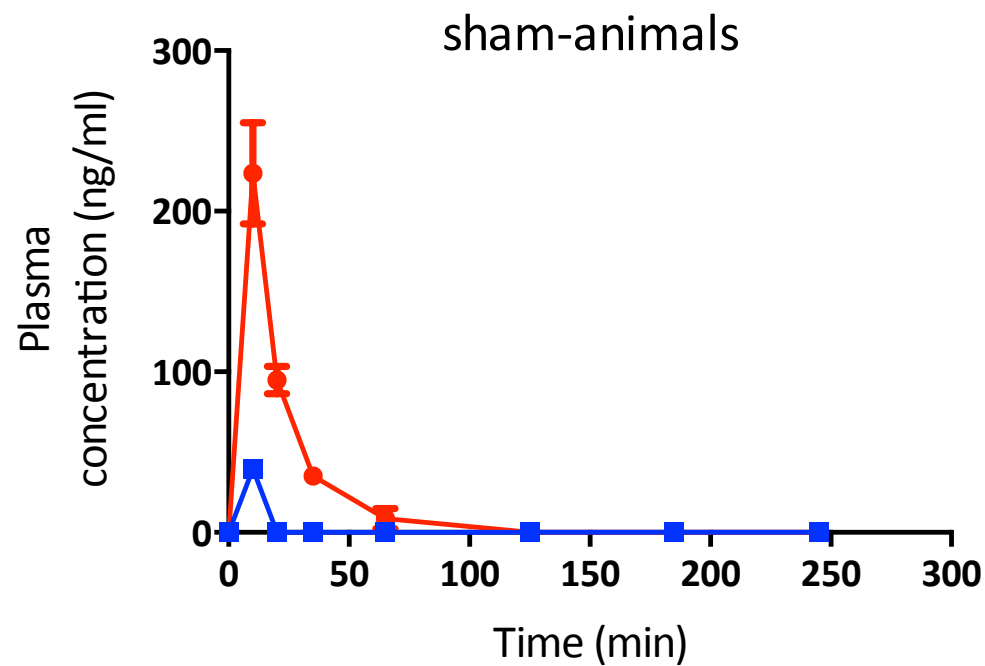
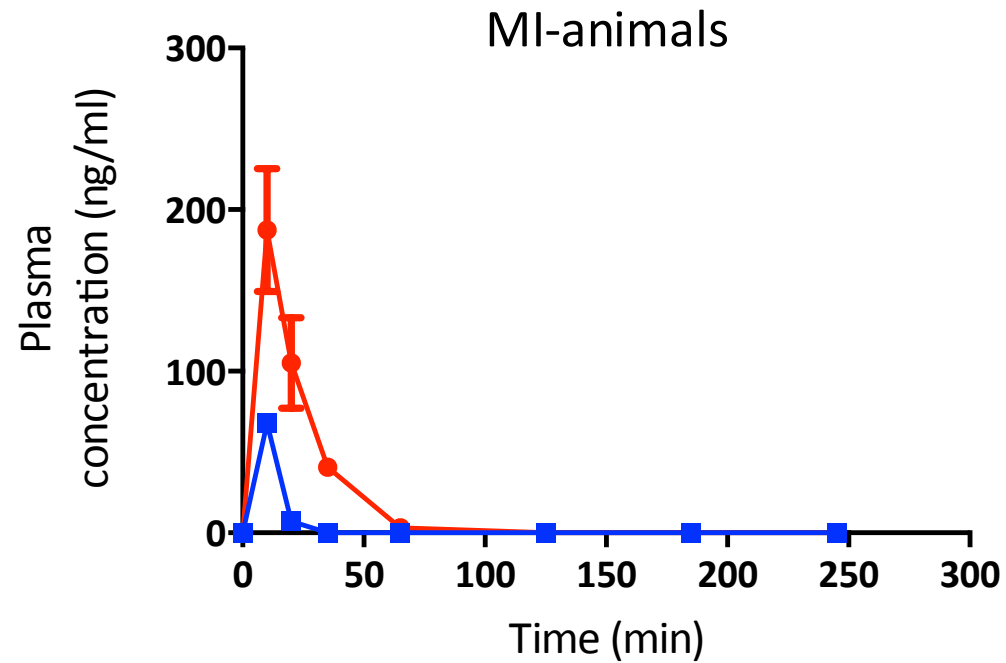


Figure 2



Artesunate



Dihydroartemisinin

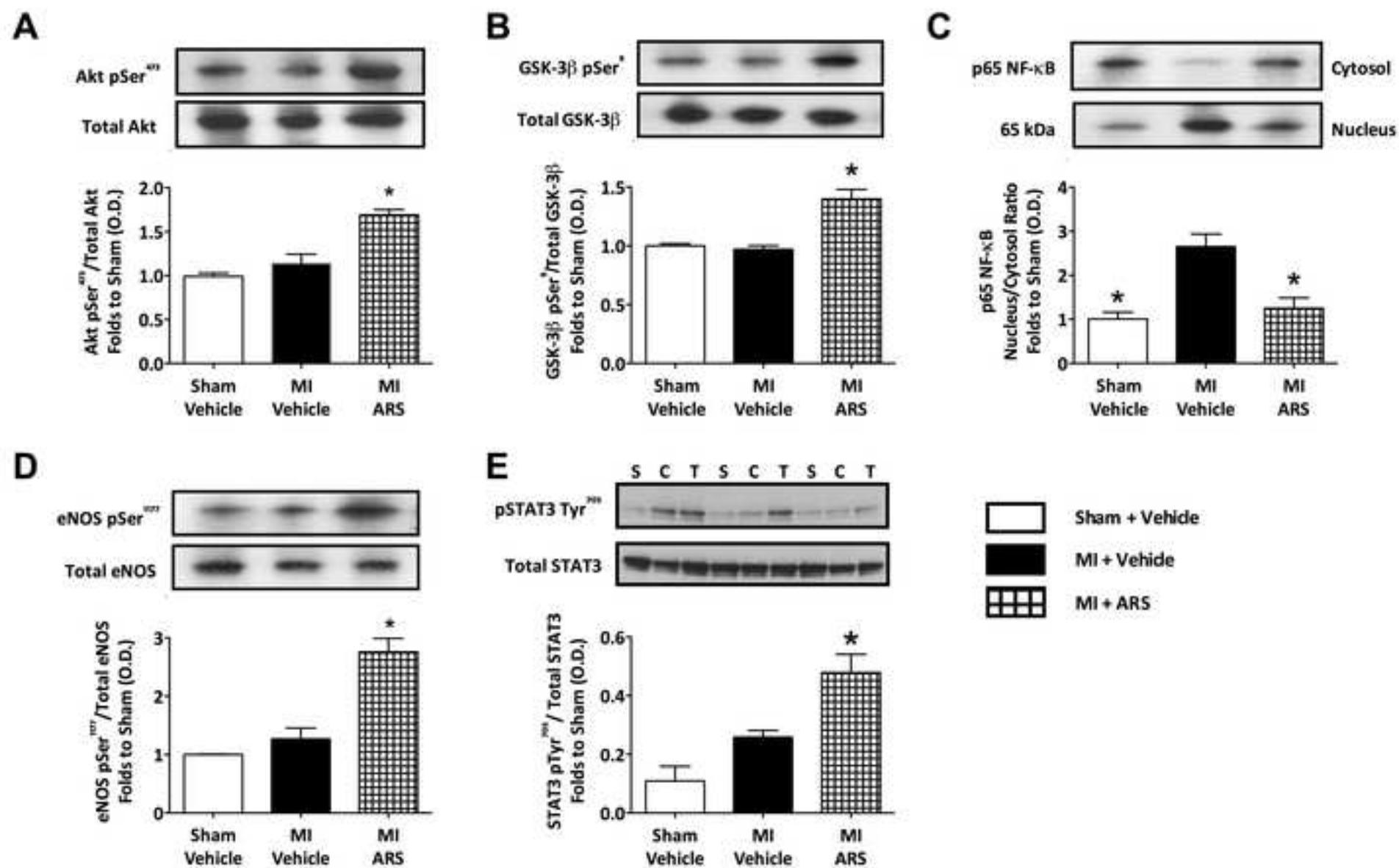


Figure 4

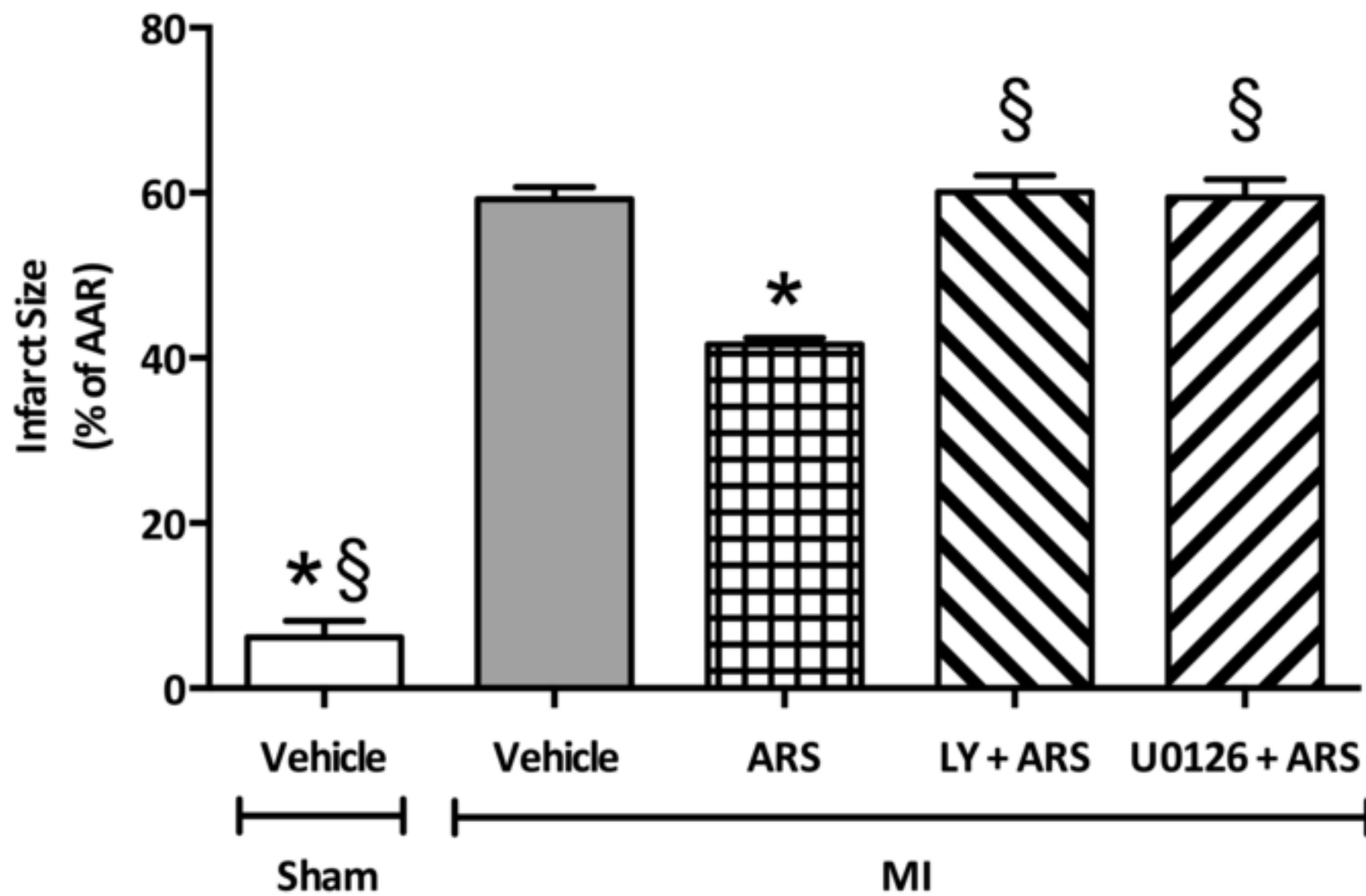


Figure 5

