Cardioprotective effects of Nitric Oxide-Aspirin in myocardial ischaemia-reperfused rats

Yilong Fu¹, Zhongjing Wang¹, Woei Lee Chen¹, Philip K. Moore¹, Yi Zhun Zhu¹,²*

¹NUS Cardiovascular Biology Research Group and Dept. of Pharmacology, National University of Singapore, Singapore 117597. School of Pharmacy and Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China.

*Corresponding author:
Dr. Yi Zhun Zhu
Dept. of Pharmacology
National University of Singapore
Singapore 117597
Tel: +65-6-874-3676
Fax: +65-6-873-7690
Email: phczhuyz@nus.edu.sg or zhuyz@shmu.edu.cn
ABSTRACT

In this study, the cardioprotective effects of the NO-Aspirin, the nitro-derivative of aspirin, were compared with that of aspirin in an anesthetized rat model of myocardial ischemia/reperfusion. Rats were given aspirin or NO-Aspirin orally for 7 consecutive days prior to 25 min of myocardial ischemia followed by 48 h of reperfusion (MI/R). Treatment groups included vehicle (Tween 80), aspirin (30mg/kg/day) and NO-Aspirin (56 mg/kg/day). NO-Aspirin, compared to aspirin, displayed remarkable cardioprotection in rats subjected to MI/R by the mortality rate and infarct size. Mortality rate for vehicle (n=23), aspirin (n=22) and NO-Aspirin groups (n=22) were 34.8%, 27.3% and 18.2%, respectively. Infarct size of the vehicle group was 44.5±2.7% of the left ventricle (LV). In contrast, infarct size of the LV decreased in the aspirin and NO-Aspirin pretreated groups, 36.7±1.8% and 22.9±4.3% respectively (both p < 0.05 compared with vehicle group, and p < 0.05 when NO-Aspirin vs. aspirin ) respectively. Moreover, NO-Aspirin also improved ischemia/reperfusion-induced myocardial contractile dysfunction on post-ischemic left ventricular developed pressure. In addition, NO-Aspirin downregulated iNOS (0.37 fold, p<0.01) and COX-2 (0.61 fold, p<0.05) genes expression compared to the vehicle group after 48 h reperfusion. Treatment with N\(^{\text{G}}\) –nitro-L-arginine methyl ester (L-NAME, 20mg/kg), a non-selective NOS inhibitor, aggravated myocardial damage in terms of mortality and infarct size, but attenuated in co-administered with NO-Aspirin. L-NAME administration did not alter the increase in iNOS and COX-2 expression but did reverse the NO-Aspirin-induced inhibition of expression of the two genes. The beneficial effects of NO-Aspirin appeared to be derived largely from NO
moiety which attenuated myocardial injury to limit infarct size and better recovery of LV function following ischemia and reperfusion.

**Key words:** cardioprotection ischemia-reperfusion, nitroaspirin, aspirin, infarct size, cardioprotection
INTRODUCTION

Ischemic myocardial tissue will, inevitably, induce necrosis if blood flow is not restored immediately. Early reperfusion after coronary obstruction is well established to recover injured myocardium; nevertheless reperfusion itself is believed to bring about additional cellular injury (25, 30). During the last two decades, numerous studies have been done which focus on the roles of nitric oxide in the pathogenesis progress and pharmacological intervention of the myocardial ischemia and reperfusion. Of them, exogenous nitric oxide (NO) donor may provide therapeutic benefit and is also a recent conceptual advance in the management of reperfusion damage (1, 17, 19, 39). However, conventional NO donor (e.g. organic nitrates) frequently results in unwanted haemodynamic effects due to the NO fast-releasing property and development of clinical tolerance. Aspirin (acetylsalicylic acid), the most widely prescribed drug in the world for the treatment of inflammatory conditions, inhibits COX in platelets, and therefore decreases thromboxane A₂ and prostacyclin (PGI₂) formations in infarcted heart. Although decreased thromboxane A₂ synthesis is beneficial, a decline in PGI₂ production is undesirable because PGI₂ possesses cardioprotective action by inhibiting platelet aggregation (50).

Recently, a new family of NO donor by linking an NO donating moiety to nonsteroidal anti-inflammatory drugs (NSAIDs) to improve organ tolerability and add new pharmacological profiles by NO slow-releasing property has been extensively investigated (21). One of these compounds, 2-acetoxy-benzoate 2-[2-nitroxy-methyl]-phenyl ester, NO-Aspirin, has been shown to be easily hydrolyzed by ubiquitous esterases in vivo into acetylsalicylic acid and unchanged NO-donating moiety in the circulatory system (6), then NO release at a constant rate from NO-Aspirin metabolite in
the same cell compartments as the endogenous NO derived from L-Arginine at the cellular level (11). NO can inhibit platelet aggregation (34) thus counteracting the inhibition of PGI2 by the aspirin moiety. Moreover, anti-inflammation effects of NO-Aspirin have been studied in vitro and in animal models. It has been found to involve in the inflammatory process of several cells (such as platelets, monocytes/macrophages, leukocytes, endothelial cells, smooth muscle cells) and interact with different inflammatory targets (3). Moreover, this compound exerts anti-arrhythmias and infarct-limiting activity through inhibition of neutrophil invasion and platelet aggregation in normal/diabetic rats and pigs following myocardial ischemia/reperfusion (MI/R) (5, 36, 46). However, these cardioprotective effects against postischemic reperfusion injury are limited to 2-3 h and employing triphenyltetrazolium chloride (TTC) staining to assess infarcted myocardium. To our interests presently, if NO-Aspirin only delayed the progression of reperfusion injury during early reperfusion in the studies reported (5, 36, 46), because, to our knowledge, rats subjected to acute myocardial infarction (AMI) are always developing severe inflammation after post-surgical few hours and reaching maximum injury/mortality in the next morning. Furthermore, in the setting of brief ischemia followed by reperfusion, whether the reduction in TTC negative tissue observed in early reperfusion signifies genuine reduction of eventual infarct size following extended reperfusion. Because the stained myocardium consists of a complex mixture of necrotic and surviving myocytes in the early reperfusion, at that time, the method of TTC staining has the limitations in the accuracy with evaluation of cell necrosis (24); furthermore, iNOS induced by pro-inflammatory cytokines occurring late phase of postischemic infarction could increase infarct size (47-49).
Our preliminary study (data not shown) showed that NO-Aspirin daily treated rats for two weeks (one week before acute myocardial infarction (AMI) and one week treatment continued after AMI; the same dosage as this study) displayed some, but not significant, cardioprotection compared to control group, the beneficial effects as evidenced by decreased infarct size/mortality to some extents, however NO-Aspirin appeared to exacerbate cardiac dysfunction: a significantly higher hypertrophy index occurred than control group ($p<0.05$). We hypothesized that this is likely NO accumulation during the myocardial infarction due to the excessive supplement of NO released from the NO moiety plus large amount NO induced from endogenous iNOS, where high concentration of NO is believed to be detrimental to cardiac tissue (8, 44, 48). Interestingly, Liang et al. demonstrated that L-Arginine (substrate for NO production) administered at different time points during ischemia/reperfusion exerted different effects on post-ischemic myocardial injury (22). Thus in the present study, we limited the treatment period of NO-Aspirin prior the induction of MI/R and evaluated its cardioprotective effect by infarct size and left ventricular function recovery at an extended reperfusion time (48 h) compared with those in vehicle and aspirin group against lethal reperfusion injury as well as the possible biochemical and molecular mechanisms involved. Endogenous NO production blocked by NOS inhibitor to define NO role from exogenous NO donor in ischemia-reperfused rats.

**MATERIALS AND METHODS**

**Animals and drug administration**
148 male, Wistar rats (200-250g) were obtained from the Laboratory Animal Centre, National University of Singapore (NUS). Animals were housed under standard conditions and maintained diurnal 12 h light/dark cycle and fed standard rat chow and water *ad libitum* according to regulations of animal care by NUS and the project conforms to the ‘Principles of laboratory animal care’ (NIH publication no. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) which were approved by the animal ethics committee of NUS.

The rats were randomly assigned to six different treatment groups, namely: group 1 (sham, n=14), sham-operated animals, thoracic open without left descending coronary artery (LCA) occlusion, treated with Tween 80 (vehicle); group 2 (vehicle + MI/R, n=23), vehicle-treated animals subjected to 25 min of LCA occlusion followed by 48h of reperfusion; group 3 (ASA + MI/R, n=22), aspirin (30mg/kg/day) treated animals and subjected to MI/R; group 4 (NOA + MI/R, n=22), NO-Aspirin (56mg/kg/day, NicOx Ltd, Sophia-Antipolis, France) treated animals and subjected to MI/R, the dose of NO-aspirin was chosen to provide a dose equimolar with that of aspirin employed and based on reports showing cardioprotective effects at this dose (36, 46); group 5 (L-NAME + MI/R, n=23), NG-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg/day), a nonspecific inhibitor of endogenous NOS, treated animals and subjected to MI/R and group 6 (NOA + L-NAME + MI/R, n=20), NO-Aspirin (56mg/kg/day) treated animals followed 1h later by L-NAME (20 mg/kg/day) and subjected to MI/R, all drugs dissolved in Tween 80, were administered orally (2ml/kg) once a day for 7 consecutive days, on the 7th day, the
last dose of drugs were administered 3 h before starting the surgical operation. The tissue samples were collected 48 h after MI/R (day 9) for biochemical and molecular studies.

Animal model of myocardial ischemia-reperfusion (MI/R)

The procedure of animal heart exposure was introduced as described earlier (16, 52, 54). In brief, under anaesthesia, rats were intubated with a cannula which connected to a rodent ventilator, and artificially ventilated with room air. The rectal temperature was monitored and core temperature was maintained between 36.7°C and 37.3°C by placing on a controlled heating pad throughout the experiment. The left thoracotomy was performed at the third and fourth ribs, the ribs were gently spread using a small-sized retractor, and then the heart was exposed. For temporary occlusion of the left descending coronary artery (LCA), a 6/0 silk suture (Ethibond, Ethicon, Norderstedt, Germany) was placed around the artery 2-3 millimeters from the aortic root, The ends of the ligature were threaded through a polyethylene tube to produce a snare. After the completion of the surgical procedure, the heart was returned to its normal position in the thorax. After 20 min of stabilization, regional ischemia was induced by tightening the snare against the ventricular wall and successful ischemia was verified by change in the color of the myocardium of left ventricle. After 25 min of ischemia, the snare was released and myocardial reperfusion was verified by change in the color of the ventricular myocardium. The thoracic cavity was then closed in layers by using 3/0 silk suture. Animals were sacrificed after 48 h for biochemical and histological studies. Hearts were collected and immediately immersed in liquid nitrogen and stored at -80°C for further studies.
**In vivo haemodynamic and regional LV function measurement**

**Systemic blood pressure**

Blood pressure (BP) using the NIBP (Non-invasive Blood Pressure) System (ML125/R, *ADInstruments Powerlab System, USA*) and electrocardiogram (ECG) monitor applying the Animal BioAmp amplifier (ML 136, *ADInstruments Powerlab System, USA*) as described (52). BP and ECG readings were measured three times per randomly-selected rat prior to the start of the treatment on day 1, subsequently prior to the surgical operation on day 7, the last pair of BP and ECG measured prior to the sacrifice of the animal on day 9.

**Left ventricular function measurements**

Animals were anesthetized after BP measurement on day 9 and artificially ventilated being placed on controlled heating pads and core temperature maintained between 36.7°C and 37.3°C. Left ventricular haemodynamics were measured by a pressure transducer (*MLT 844 high precision BP transducer, AD Instruments, California*). After stabilization for 20 min, the pressure signal was continuously recorded using a *MacLab A/D converter (AD Instruments, Mountain View, California)* and stored and displayed on a computer. The peak left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDevP, expressed as the difference between systolic and diastolic pressures) were measured and the maximal slopes of systolic pressure increment (LV dP/dt<sub>max</sub>) and diastolic pressure decrement (LV dP/dt<sub>min</sub>), indexes of contractility and relaxation, were analyzed.
Infarct size determination

Infarct size was identified by 2,3,5- Triphenyltetrazolium chloride (TTC) as we previously reported (16). In brief, the infarcted area was judged from both epicardial and endocardial sides and outlined on paper, cut and weighed. The infarct size is defined as a ratio of the left ventricular infarct area to the whole left ventricle area (41). The sizes of the left ventricle and the infarct area were evaluated by software of Scion Image (Scion Inc., California, USA).

Measurement of plasma nitrate/nitrite

Nitrate/nitrite (NOx) was determined in plasma collected immediately at the end of 48 h after surgery. Briefly, intracardiac blood samples (0.5 ml) were collected. The blood was centrifuged for 15 min at 2,400g at 4°C and the plasma supernatant was removed and stored frozen at -80°C until assayed. NOx was determined spectrophotometrically in aliquots (80 µl) of plasma using the Greiss reagent as described (52). In brief, aliquots of plasma were incubated (37°C, 30 min) with nitrate reductase (10 mU) in the presence of NADPH (100 µM) to reduce nitrate to nitrite and then centrifuged (14,000 g, 25 min, 4°C). The resulting supernatant and sodium nitrite standard (100 µl; 0.125–75 µM) were added in duplicate to 96-well microtiter plates. Thereafter, Greiss reagent (containing 0.2% N-1-naphtyl ethylenediamine dihydrochloride and 2% w/v sulfanilamide in 5% v/v H₃PO₄) was added into the above mixture in a ratio of 1:1 (v/v) and incubated for 10 min at room temperature after which absorbance was determined at 550 nm in a 96-well microplate reader (Tecan Systems Inc.). The concentration of nitrite (indicative of NOx
in the original samples) was calculated from a standard curve of NaNO₂ (0.125–75 µM) and expressed as micromolar nitrite.

**RNA extraction and reverse transcriptase – polymerase chain reaction (RT-PCR) amplification**

Total RNA was extracted according to standard protocol (53). 1µg total RNA of each sample was reverse-transcribed into first-strand complementary DNA (cDNA) and amplified using one step reverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen, Germany). Briefly, 1µg of RNA from each pooled sample was used in RT-PCR. The RT-PCR was carried out in a total volume of 20µl, containing 4µl of Qiagen OneStep RT-PCR buffer, 0.8µl dNTP, 1.2µl of sense primer, 1.2µl of anti-sense primer, 0.8µl Qiagen OneStep RT-PCR Enzyme Mix. RT-PCR was carried out in a thermocycler (GeneAmp PCR System 2700). Firstly, the samples were incubated at 50°C for 30 minutes to allow reverse transcription for the synthesis of cDNA. Following, the samples were subjected to PCR amplification using primers specific for COX-1, COX-2, eNOS, nNOS, iNOS and GAPDH. Three-step PCR of denaturing and extension were carried out at 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 30 seconds respectively. The PCR products were in linear and not yet reached plateau. The annealing temperature and PCR cycle for different gene were set as follow: GAPDH (55°C for 30 seconds, 25 cycles) and COX-1, COX-2, eNOS, nNOS, iNOS (55°C for 30 seconds, 35 cycles). The primer sequences and their product sizes are given in Table 1.

**Statistical analysis**
Statistical comparisons between groups were analyzed using One-way analysis of variance (One-way ANOVA) and post hoc unpaired, 2-sided Student’s t test with a Bonferroni adjustment. All data were presented as mean ± SEM. Significant differences among the groups were defined by a \( p \) value of less than 0.05.

**RESULTS**

**Mortality and infarct size**

The results of the infarct size and mortality in MI/R rats are reported (Table 1). Mortality rate for vehicle (n=23), aspirin (n=22) and NO-Aspirin (n=22) treated groups were 34.8%, 27.3% and 18.2% respectively. Although no significant difference when compared any two groups, NO-Aspirin and aspirin tended to reduce the mortality rate and more rats in NO-Aspirin treated group survived than those in aspirin treated group. In contrast, L-NAME had a higher mortality rate (45.0%) than vehicle treated group (34.8%); however, co-administration of NO-Aspirin with L-NAME, the higher mortality of L-NAME was abrogated (34.8%).

There were no significant differences in body weight (BW), heart weight (HW) and left ventricle weight (LVW) among all groups, corresponding reduction in infarction size in the groups with better survival rate was noted. Thus, infarction size (n=5) for the vehicle group was 44.5%± 2.7% of the left ventricle (LV) while the infarction size for aspirin and NO-Aspirin groups was reduced to 36.7%± 1.8% and 22.9%± 4.3% of the LV respectively. Infarct sizes were significantly decreased in both aspirin and NO-Aspirin treated groups as compared to the vehicle group (\( p < 0.05 \)). There was a significant difference observed between aspirin and NO-Aspirin-treated groups (\( p < 0.05 \)). Compared
to vehicle group, treatment of L-NAME increased infarct size \( (p < 0.05) \) and completely blunted the NO-Aspirin induced reduction in infarct size when combined treatment.

**Haemodynamic measurements**

*Systemic blood pressure and heart rate*

6-8 rats from each treatment group were randomly selected for the measurement of systemic BP. Systemic blood pressure (systemic BP) and heart rate (HR) were measured (Table 2) at 3 time points: just prior to starting drug treatment (baseline), at 1 h after last drug treatment on day 7, and at 48 h after the onset of reperfusion (end of reperfusion). Baseline BP values were in the same range in all treatment group. After 7-day treatment, BP in NO-aspirin treated group showed a slight but not significant decrease compared to that in vehicle group.

In L-NAME treated group, systemic BP significant increase after 7-day treatment but marked decrease at the end of 48 h reperfusion observed compared with vehicle group \( (p < 0.01) \). In L-NAME co-administered with NO-Aspirin, NO-Aspirin abolished L-NAME-induced systemic BP increase (not significant, N.S.) and attenuated L-NAME-induced BP decrease at 48 h reperfusion \( (p < 0.05 \text{ in NO-Aspirin vs. } p < 0.01 \text{ in L-NAME group}) \).

Baseline HR were in the same range and remained unchanged after treatment and at 48 h reperfusion in rats treated with vehicle, aspirin and NO-Aspirin. L-NAME had no effect on HR too.

*Left ventricle mechanic function parameters*
Figure 1 and Table 3 depict the left ventricular mechanic function parameters at 48 h reperfusion including LVDevP, LVSP, LV dP/dt\textsubscript{max} and LV dP/dt\textsubscript{min}. Compared with sham group, significantly decreased on LVDevP, LV dP/dt\textsubscript{max}, LV dP/dt\textsubscript{min} were noted in rats subjected to MI/R. Figure 1 depicts that NO-Aspirin improved significantly LVDevP from reperfusion injury compared to sham group (N.S.) while other treatment have no such influence ($p<0.05$ vs sham group). NO-Aspirin and aspirin appeared to increase, although not significantly, LVSP, LV dP/dt\textsubscript{max} and LV dP/dt\textsubscript{min} (N.S.) compared with vehicle group, which demonstrates that the left ventricular mechanic function recovered partly from reperfusion injury (Table 3). However, L-NAME tended to decrease LVSP, LV dP/dt\textsubscript{max} and LV dP/dt\textsubscript{min} as compared to vehicle group, and NO-Aspirin combined with L-NAME exerted similar results as L-NAME.

**Plasma nitrite/nitrate (NOx) concentration**

NOx concentration which is the stable end product of NO in plasma is shown in Figure 2. Treatment for 7 consecutive days with NO-Aspirin caused a marked increase of plasma NOx ($p<0.05$ vs sham and vehicle group). Rats subjected to MI/R (vehicle group, 33.3$\pm$1.5 $\mu$M) showed a higher NOx level compared to sham group (17.6$\pm$2.4 $\mu$M, $p<0.05$). Aspirin (25.6$\pm$2.1 $\mu$M), NO-Aspirin (17.3$\pm$1.6 $\mu$M) showed attenuation of NOx level compared to vehicle group, but significant difference only occurred in NO-Aspirin treated group ($p<0.01$).

**Gene expression of NOS and COX**
The gene expressions for NOS and COX are shown in Figure 3 (A-E). All drug treated groups had no significant influence on COX-1, eNOS and nNOS mRNA expression compared to those in vehicle treated animals after 48 h reperfusion. The level of iNOS mRNA expression increased significantly in vehicle group compared to that in the sham group (2.6-fold), but compared with that in the vehicle group, it decreased in Aspirin (0.64-fold, \( p<0.05 \)) and NO-Aspirin (0.37-fold, \( p<0.01 \)) groups. The COX-2 mRNA level increased significantly too in vehicle group compared to sham group (2.5-fold). There were significant lower levels of COX-2 production in aspirin, NO-Aspirin treated group (\( p<0.01 \)) compared to the vehicle group. L-NAME administration did not alter the increase in iNOS and COX-2 expression but did reverse the NO-Aspirin-induced inhibition of expression of these genes.

**DISCUSSION:**

The present results clearly demonstrated that the 7-consecutive-day oral administration of NO-Aspirin before myocardial ischemia could offer noticeable cardioprotection by significantly limiting infarct size/lowering mortality rate caused by 25 min LCA occlusion followed 48 h reperfusion, which confirmed previous infarct-limiting effect during early reperfusion (5, 36, 46); moreover, NO-Aspirin-improved part of cardiac dysfunctional recovery was noted at the 48 hour of reperfusion.

**Cardioprotection of pretreatment NO-Aspirin against cell damage**

In the present study, compared with equimolar dose of aspirin treated animals, NO-Aspirin displayed marked infarct-limiting effect, the mechanism(s) involved probably is
that enhanced NO bioavailability initiates a preconditioning (PC)-like phenomenon. Based on previous studies reported, exogenous NO (NO donor) could trigger/mediate cardiac adaptive protection against reperfusion injury from first 2-3 h to a few days (28, 33, 43), although the exact targets or effectors of NO in PC are open to investigation (17). Given the multifarious nature of NO’s chemical and physiological actions, supplement with NO could also exert inhibition of platelet aggregation and neutrophil adhesion during reperfusion (34, 36, 37), we believe that the enhanced NO bioavailability by NO-Aspirin pretreatment elicit salubrious effects. Of note, NO-Aspirin was not sufficient to cause any appreciable systemic BP and heart rate change compared to baseline level, which might be due to the NO slow-releasing property; therefore, any difference in NO-Aspirin treated group cannot be attributed to haemodynamic alterations.

Pretreatment of L-NAME, the non-selective NOS inhibitor, before ischemia increased systemic BP compared to baseline level and exacerbated the extent of myocardial reperfusion injury compared to vehicle group, but co-administration of NO-Aspirin attenuated the hypertensive state and worsening effect in term of infarct size/mortality rate induced by L-NAME, these findings also agree with previous studies (27, 36). The mechanism is likely that exogenous NO supplement constitute the reduced endogenous NO production by chronic L-NAME treatment before MI/R. In the normal physiological condition, endothelial NO synthase (eNOS) displayed more infarct-limiting effect than neuronal NO synthase (nNOS) did by comparing the infarct size among the wild-type, eNOS and nNOS knockout mice subjected to global MI/R (40). Jones et al also demonstrated that endothelial cell-derived NO played a vital role in vascular homeostasis.
within the coronary circulation and displayed the intrinsic defenses against MI/R-induced cardiomyocyte injury in eNOS knockout mice (18). The beneficial effects of eNOS was supported by applying transgenic (TG) mice that overexpress eNOS exclusively in cardiac myocytes, these authors reported that high levels of NO/cGMP strongly protect against ischemia/reperfusion injury related to reduced preischemic performance than wild-type mice (40). NO-Aspirin, unlike conventional NO donors, releases NO intracellular at a rate similar to that generated by endogenous eNOS (12, 14, 20). Thus, we propose that NO donated by NO-Aspirin maintain or augment eNOS-exerting vasodilatory tone and defensive functions against reperfusion injury, and abolish the endothelial dysfunction and injury exacerbation by L-NAME when combined administration with NO-Aspirin.

**Cytotoxicity of iNOS-derived NO exacerbates LV dysfunction**

Rossini et al reported that NO-Aspirin did not change the cardiac mechanic parameters (LVP, LVDevP, heart rate) in rats subjected to 2 h reperfusion following myocardial ischemia (36), and our preliminary results showed that hypertrophy occurred in NO-Aspirin treated rats after AMI, however our present study demonstrates NO-Aspirin recovered LV function partly at 48 h after reperfusion, but the decrease of infarct size in NO-Aspirin treated groups were observed in all the three studies. Moreover, it is interesting in the present study that plasma total NOx measured at 48 h reperfusion was significantly lower treated with NO-Aspirin compared to that of vehicle-treated animals in spite of 7-day NO supplement. We speculate that iNOS-derived NO plays a vital role
in NO accumulation during 2 days reperfusion and also results in the discrepancy of LV function among the three studies.

Different from constitutive NOS (eNOS and nNOS), iNOS can be induced by pro-inflammatory substances such as cytokines and lipopolysaccharide (LPS) or pathological conditions such as stroke, trauma, infection and a variety of cardiovascular diseases (13) and produces higher levels of NO than the normal for a longer period (32); Wildhirt et al reported that iNOS activity increased and reached maximal level range from 48-72 h after initiation of AMI (48) and MI/R in rabbits (49), which was also supported by significant augments of plasma NOx level and cardiac iNOS mRNA expression in control group than those of sham group. Although considerable evidences exist that NO itself has low reactivity with most biological molecules and is cytoprotective, large amount of NO produced by iNOS is recognized as a mediator and regulator of inflammatory responses (39, 55). Because overproduction of NO can combine with superoxide to form the potent oxidant peroxynitrite (ONOO\textsuperscript{-}) in which rate is far exceeding than endogenous SOD compete with, peroxynitrite is a highly cytotoxic molecule that contributes to cardiac dysfunction and myocardial injury under a variety of cardiovascular diseases, including MI/R(10, 35); inhibition of iNOS can recover the LV function and protect the salvaged reperfused myocardium against post infarction dysfunction (48, 49).

Therefore, the timing of exogenous NO supplement, the enzymatic source of endogenous NO generation in physiological / pathological condition and the concurrent formation of the toxic reaction product between NO and reactive oxygen species (ROS) could explain the discrepancy between the present and the preliminary study against cardiac mechanic dysfunction. Of note, whether NO released from NO-Aspirin interacted with the
endogenous NOS before and during the MI/R needs further investigation, which is beyond the scope of the present study.

**COX-2 expression**

Another point of interest is to evaluate COX isoforms mRNA level at 48 hour reperfusion. The two distinct COX isoforms, namely COX-1 and COX-2, have been characterized. COX-1 is constitutively expressed in most cells, mostly in platelets and endothelial cells and is responsible for homeostatic prostanoid formation, and COX-2, which is almost undetectable or lower in basal conditions, is dramatically upregulated in respond to stress, such as inflammatory cytokines and ischemia, and often involves in harmful effects including trauma, LPS induced inflammation and I/R injury. Several reports have suggested that the inhibition of COX-2 ameliorates tissues (lung, kidney and liver) damage triggered by I/R injury (9, 15, 51). Oshima et al. (31) reported that inhibition of COX-2( FK3311) significantly improved cardiac function in dogs following transplantation. Bouchard et al. (4) showed that COX-2 inhibitors protected the endothelial function against the deleterious effect of ischemia and reperfusion in Isolated rat hearts. Therefore, we deduced that pretreatment with NO-Aspirin may attenuate COX-2 expression, and in turn, contribute to ameliorating cardiac malfunction and damage in this study.

Another reason to investigate COX-2 mRNA expression is that COX-2 closely related with iNOS. First, COX-2 is found to be co-induced together with iNOS in cardiac myocytes and many other cell types during inflammatory cytokines and ischemia (23, 29,
Furthermore, the signaling molecules that induce the expression of COX-2 in response to stress similar to those that activate iNOS expression, which including nuclear factor-kappa B (NF-κB) (7, 38) and protein kinase C (PKC) (2, 26). The COX-2 mRNA expression results validate our hypothesis: NO-Aspirin exhibited a significant decrease of COX-2 mRNA expression, just like result in decrease of iNOS mRNA, than vehicle (p < 0.01). As a consequence of our study, pretreatment NO-Aspirin attenuated MI/R injury by possibly blunting COX-2 induction during reperfusion.

In conclusion, NO-Aspirin exerts a better cardioprotective effects than aspirin in the rat model of ischemia and 48 h reperfusion, which attenuated myocardial injury evidence as infarct-limiting and partly left ventricular function recovery following ischemia and reperfusion. Thus, the beneficial effects of NO-Aspirin to decrease iNOS and COX-2 mRNA levels seems to involve a NOS-dependent pathway. It also needs to be explored whether other mechanisms or molecular targets of NO are involved in the protection of postischemic injury. The findings of this study, however, offer a new possibility for NO-Aspirin pretreatment in management of reperfusion injury. The cardioprotective effect of NO-Aspirin would also benefit the aged and endothelial-function impaired patients for whom myocardial dysfunction is a common life-threatening complication.

ACKNOWLEDGEMENTS

This work was supported by a grant R-184-000-082-213 from the National Medical Research Council of Singapore and a block grant (R-184-000-074-712) of Office of Life Sciences, National University of Singapore.
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TABLES

**Table 1.** Mortality, body weight (BW), heart weight (HW), left ventricle weight (LVW), infarct size in rats subjected to 25min of coronary artery occlusion followed by 48 h of reperfusion. Values are means ± SE \(^a\) \(p<0.05\) compared to vehicle treated group \(^b\) \(p<0.05\) compared to Aspirin treated group.

**Table 2.** Systemic blood pressure (BP) and heart rate (HR) in rats prior to experiment on day 1 (baseline), day 7 (after 7 consecutive days of treatment) and day 9 (48 h after reperfusion). Values are means ± SE, n=6-8 for each group \(^a\) \(p<0.05\) compared to baseline \(^b\) \(p<0.01\) compared to baseline.

**Table 3.** \(LVSP, \ LV \ dP/dt_{max}, \ LV \ dP/dt_{min}\) in rats subjected to 25min of coronary artery occlusion followed by 48 h of reperfusion. Values are means ± SE, n=5-7 for each group \(^a\) \(p<0.05\) compared to sham group \(^b\) \(p<0.01\) compared to sham group.

**Table 4.** Gene sequences and corresponding product sizes of GADPH, COX-1, COX-2, iNOS, nNOS, eNOS.
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<th>BW (g)</th>
<th>HW (g)</th>
<th>LVW (g)</th>
<th>Infarct size % of LV</th>
<th>Mortality(n)</th>
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<td>Sham</td>
<td>259.5±4.3</td>
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<td>0.490±0.022</td>
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<td>0.504±0.020</td>
<td>36.7±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.3(22)</td>
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<td>NO-Aspirin</td>
<td>244.5±4.3</td>
<td>0.808±0.040</td>
<td>0.514±0.035</td>
<td>22.9±4.3&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>18.2(22)</td>
</tr>
<tr>
<td>NOA+ L-NAME</td>
<td>248.0±10.3</td>
<td>0.838±0.031</td>
<td>0.532±0.020</td>
<td>42.0±4.2</td>
<td>34.8(23)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>257.9±7.5</td>
<td>0.796±0.030</td>
<td>0.464±0.030</td>
<td>55.0±2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.0(20)</td>
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### Table 2

<table>
<thead>
<tr>
<th></th>
<th>day1</th>
<th>day7</th>
<th>day9</th>
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<tbody>
<tr>
<td><strong>Systemic BP (mmHg)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>119.0±3.9</td>
<td>123.7±3.4</td>
<td>116.6±3.4</td>
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<tr>
<td>Aspirin</td>
<td>123.9±3.6</td>
<td>119.0±3.8</td>
<td>106.8±4.9</td>
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<tr>
<td>NO-Aspirin(NOA)</td>
<td>122.4±4.9</td>
<td>113.7±3.2</td>
<td>111.8±4.6</td>
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<tr>
<td>NOA+ L-NAME</td>
<td>124.5±4.4</td>
<td>128.7±4.1</td>
<td>104.2±1.7a</td>
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<tr>
<td>L-NAME</td>
<td>124.2±5.1</td>
<td>138.4±1.0a</td>
<td>97.5±3.5b</td>
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<td><strong>HR (Beat/min)</strong></td>
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<tr>
<td>Vehicle</td>
<td>425±13</td>
<td>434±15</td>
<td>431±13</td>
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<tr>
<td>Aspirin</td>
<td>451±14</td>
<td>426±19</td>
<td>445±7</td>
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<tr>
<td>NO-Aspirin(NOA)</td>
<td>447±11</td>
<td>445±10</td>
<td>432±10</td>
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<tr>
<td>NOA+ L-NAME</td>
<td>429±17</td>
<td>437±13</td>
<td>461±13</td>
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<tr>
<td>L-NAME</td>
<td>429±22</td>
<td>441±10</td>
<td>423±11</td>
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Table 3

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<th>LVSP mmHg</th>
<th>LV dP/dt max  x1000 mmHg/s</th>
<th>LV dP/dt min x1000 mmHg/s</th>
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<td>sham</td>
<td>103.5±4.9</td>
<td>4.7±0.4</td>
<td>3.0±0.2</td>
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<td>Vehicle</td>
<td>92.3±3.6</td>
<td>3.1±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Aspirin</td>
<td>89.1±5.4</td>
<td>3.5±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.3</td>
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<tr>
<td>NO-Aspirin(NOA)</td>
<td>96.4±1.5</td>
<td>3.3±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.2</td>
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<tr>
<td>NOA + L-NAME</td>
<td>87.2±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>L-NAME</td>
<td>85.0±4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
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Table 4

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<th>Gene</th>
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<td>COX-1</td>
<td>5’-CGAGGATGTCATCAAGGAG-3’ 5’-TCAGTGAGGCTGTGTAAACG-3’</td>
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<td>COX-2</td>
<td>5’-CTGTATCCCGCCCTGCTGGTG-3’ 5’-ACTTGCGTTGATGGTGCCCTGTCTT-3</td>
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<td>eNOS</td>
<td>5’-CTGGCAAGACCGATTACACGA-3’ 5’-CGCAATGTGAGTCCGAAAATG-3’</td>
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<td>NM_021838</td>
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<td>iNOS</td>
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<td>442</td>
<td>NM_012611</td>
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<td>nNOS</td>
<td>5’-AATGGAGACCCCCCTGAGAAC-3’ 5’-TTCAAGGAGGTTGCTCCACCGC-3’</td>
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<td>NM_052799</td>
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<td>GAPDH</td>
<td>5’-CATGGTCTCATGTCCAGTTCAT-3 5’-GGCTAAGCAGTTGGTGTCGC-3’</td>
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COX, cyclooxygenase; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS
**Figure Legend**

**Figure 1.** LVDevP(mmHg) in rats subjected to 25min of coronary artery occlusion followed by 48 h of reperfusion. Values are means ± SE. \( ^a p<0.05 \) compared to sham group

**Figure 2.** Plasma NOx concentration(\( \mu \)M) in rats subjected to 25min of coronary artery occlusion followed by 48 h of reperfusion. Values are means ± SE, n=4-6, \( ^a p<0.05 \) compared to vehicle treated group (just before MI/R). \( ^b p <0.01 \) compared to vehicle treated group (48 hour after MI/R).

**Figure 3.** Gene expressions (the gel photo represent 3 individual experiments) and quantifications of: (A) inducible (iNOS), (B) neuronal (nNOS), (C) endothelial nitric oxide synthase(eNOS), (D)COX-1 and (E) COX-2 in sham, vehicle, aspirin, NO-Aspirin, NO-Aspirin + L-NAME and L-NAME pretreated rats subjected to 25min of coronary artery occlusion followed by 48 h of reperfusion. Values are means ± SE. \( ^a p < 0.05 \) compared to vehicle treated group. \( ^b p < 0.01 \) compared to vehicle treated group.
Figure 1
Figure 2
Figure 3

Lane 1 = sham; Lane 2 = vehicle; Lane 3 = Aspirin; Lane 4 = NO-Aspirin (NOA); Lane 5 = NO-Aspirin (NOA) + L-NAME; Lane 6 = L-NAME

A)
B) RATIO of nNOS/GAPDH
C) RATIO of eNOS/GAPDH.
D) **RATIO of COX1/GAPDH**

![Error plotting graph](image-url)
E) RATIO of COX2/GADPH

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Values: b b