Cardioprotective Effects of Luteolin During Ischemia–Reperfusion Injury in Rats

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Background: Antioxidants effectively reduce ischemia–reperfusion (IR) injury. The cardioprotective effects of luteolin, a flavonoid that exhibits antioxidant properties and is widely available in many fruits and vegetables, were examined in rats subjected to myocardial IR injury.

Methods and Results: Rats were subjected to myocardial ischemia or reperfusion injury to evaluate the anti-arrhythmic effects of luteolin. Myocardial infarct size was determined histochromically with triphenyltetrazolium chloride staining of the left ventricle. Luteolin was administered intravenously 15 min before occlusion of the coronary artery. The incidence and duration of ventricular tachycardia and ventricular fibrillation and mortality during myocardial ischemia were significantly reduced by luteolin (10 μg/kg). Similarly, luteolin (1 μg/kg) reduced ventricular arrhythmias and mortality during the reperfusion phase. Pretreatment with luteolin decreased plasma lactate dehydrogenase and nitric oxide (NO) levels. Luteolin (10 μg/kg) significantly reduced the myocardial infarct size, as well as malondialdehyde production in tissue samples of myocardial IR injury. Luteolin also downregulated inducible NO synthase protein and mRNA expression, but did not significantly alter neuronal NO synthase or endothelial NO synthase expression.

Conclusions: Luteolin is capable of protecting the myocardium against IR injury. The actions of luteolin are at least partly mediated through downregulation of NO production and its own antioxidant properties. (Circ J 2011; 75: 443–450)

Key Words: Cardioprotective agent; Ischemia; Luteolin; Nitric oxide; Reperfusion

Ischemia is characterized in part by low tissue oxygen tension. It is also well documented that salvage of the ischemic myocardium is dependent upon timely reperfusion; therefore, it is likely that the very events critical for survival may, in fact, lead to further tissue injury. Evidence from various investigations of the myocardium suggests that reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen, contribute to the pathophysiology of myocardial ischemia–reperfusion (IR) injury. These ROS, which are formed within the ischemic myocardial cells and in the first few moments of reperfusion, are known to be cytotoxic to surrounding cells. Thus, myocardial IR injury induces ventricular arrhythmias, resulting in circulation collapse and sudden death. Effective inhibition of the production of ROS or elimination of oxygen-derived free radicals is therefore an important strategy for the treatment of ventricular arrhythmias and myocardial infarction (MI) caused by myocardial IR injury.

Luteolin is a widely distributed flavonoid, one of a group of naturally occurring polyphenolic compounds found in many fruits and vegetables. It has been reported in the literature that luteolin has a wide range of biological and pharmacological properties, including antineoplastic, anti-hepatotoxic, anti-allergic, antiosteoporotic, anti-diabetic, anti-inflammatory, anti-platelet and vasodilatory activity, as well as antioxidant effects. At low concentrations (IC50 of 0.96 μmol/L), luteolin has also been shown to inhibit xanthine

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The first two authors contributed equally to this work (P.-H.L. and L.-M.H.).

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oxidase activity, which has been implicated in tissue-related oxidative injury after IR.\textsuperscript{20} Recently, in human melanoma HMB-2 cells, luteolin showed concentration-dependent inhibitory activity toward DNA damage induced by H$_2$O$_2$.\textsuperscript{21} In addition, luteolin has been shown to significantly enhance left ventricular pressure and the global and relative coronary flow in Langendorff rabbit hearts subjected to repeated myocardial ischemia.\textsuperscript{22}

The antioxidant properties of luteolin prompted us to investigate whether it is capable of exerting beneficial effects during myocardial IR injury in anesthetized rats subjected to transient coronary artery occlusion. Animals were pretreated with or without luteolin before coronary artery ligation. The severity of myocardial IR-induced arrhythmias, including the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF), mortality and infarct size, were compared between the groups of animals.

**Methods**

**Animals**

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We used male Sprague-Dawley rats (National Lab. Animal Breeding and Research Center, Taipei, Taiwan) weighing 250–300 g. The animals were housed in a room under controlled temperature (24±1°C) and humidity (55±5%) conditions and subjected to a 12:12 h light–dark cycle. They were allowed free access to food and water.

**Surgical Procedure**

Myocardial IR injury was induced by temporary occlusion of the left main coronary artery in a procedure described previously.\textsuperscript{23} Briefly, the rats were anesthetized with intraperitoneal urethane (1.25 g/kg) and placed on an operating table. The trachea was cannulated for artificial respiration and the jugular vein was cannulated for drug administration. Polyethylene catheters (PE-50) were inserted into the common carotid artery for continuous monitoring of heart rate (HR) and arterial blood pressure (BP) by a Statham P23 XL transducer and data were displayed on a Gould RS-3400 physiological recorder (Gould, Cleveland, OH, USA). A standard lead-1 ECG was recorded via silver electrodes attached to the extremities.

After tracheotomy, the animals were ventilated with room air, using a respirator for small rodents (Model 131, NEMI, USA) with a stroke volume of 15 ml/kg body weight and at a rate of 60 strokes/min to maintain normal P$_{aco}$, P$_{aco}$ and pH parameters (blood gas analyzer, GEM-5300 IL, CO, USA). The chest was opened by a left thoracotomy, followed by sectioning of the 4th and 5th ribs, approximately 2 mm to the left of the sternum. The heart was quickly externalized, inverted and a 6/0 silk ligature was placed around the left main coronary artery. The heart was repositioned in the chest and the animal was allowed to recover for 15 min. Animals in which the procedure produced arrhythmia or a sustained decrease in BP to less than 70 mmHg were not included in the study.

A small plastic snare formed from a Portex P-270 cannula was threaded through the ligature and placed in contact with the heart. The coronary artery was then occluded by tightening the ligature and reperfusion was achieved by releasing the tension applied to the ligature (operated groups). Successful ligation of the coronary artery was validated by observation of a decrease in arterial pressure and ECG changes (increase in R wave and ST segment elevation) indicative of ischemia. Sham-operated animals underwent all surgical procedures, except that the silk passing around the left coronary artery was not tied.\textsuperscript{24}

**Evaluation of Arrhythmia**

For evaluating the antiarrhythmic effects of luteolin during myocardial IR injury, the coronary artery was occluded for 30 min or 5 min followed by 30 min of reperfusion. In previous studies, the majority of myocardial ischemic arrhythmias have occurred during the first 30 min of ligation,\textsuperscript{25} and a 5-min period of ischemia followed by a 30-min reperfusion is associated with the highest incidence of reperfusion-induced arrhythmias.\textsuperscript{26} Before and during the ischemia or reperfusion period, HR, BP, and ECG changes were recorded simultaneously on a personal computer with waveform analysis software (AcqKnowledge, Biopac System, Goleta, CA, USA). Ventricular ectopic activity was evaluated according to the diagnostic criteria advocated by the Lambeth Convention.\textsuperscript{27} The incidence and duration of ventricular tachyarrhythmias, including VT and VF, were determined in both survivors and those that eventually died. In rats with irreversible VF, the duration of VF was recorded until BP was <15 mmHg.

**Estimation of Myocardial Injury**

Myocardial cellular damage was evaluated by measuring lactate dehydrogenase (LDH) activity in plasma. LDH released from necrotic tissue was determined from arterial blood drawn from the carotid catheter at the end of IR injury and collected in polyethylene tubes containing 50 μl heparin (250 IU). LDH activity was measured according to the method of Tsai et al.,\textsuperscript{28} spectrophotometrically following the rate of conversion of reduced nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide at 340 nm with a commercially available assay kit (Sigma, St Louis, MO, USA).

**Plasma Levels of Nitric Oxide Metabolite (NOx)***

Arterial blood samples were drawn from the carotid catheter at the end of ischemia or reperfusion. The assay has been previously described in detail.\textsuperscript{29} Nitric oxide (NO) production was estimated from the amounts of nitrite (NO$_2^-$) and nitrate (NO$_3^-$) in deproteinized plasma samples assayed with a commercially total NO assay kit (Stressgen, Ann Arbor, MI, USA). NO$_2^-$ was calculated by first reducing NO$_3^-$ to NO$_2^-$ in the presence of cadmium, and NO$_3^-$ was determined by a colorimetric assay based on the Griess reaction. Measuring the NO$_3^-$ levels has been confirmed as a reliable method of determining the synthesizing capacity of NO synthase (NOS) in the heart.

**Estimation of Size of Myocardial Infarct**

Only those rats that survived 1 h of coronary ischemia and 3 h of reperfusion were included in the evaluation of the infarct zone. The size of both the occluded zone and infarct zone in the rats’ hearts were determined by procedures previously described by Hung et al.\textsuperscript{30} Prolonged IR duration was required to produce an infarct suitable for pathological evaluation. At the end of the experiment, the coronary artery was re-occluded and 2.0 ml 3% methyl blue was injected into it. With this technique, the previously non-ischemic area appears blue while the area at risk remains unstained. The latter region was excised, weighed and the occluded zone was expressed as the percentage of the total ventricular weight. Thereafter, ventricular tissue was sliced into 1-mm sections and incubated in tetrazolium dye (2,3,5-triphenyltetrazolium chloride 1% (Sigma, USA) in normal saline) at 37°C for 40 min in
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darkness. Sections were then placed in a solution of 10% formaldehyde in saline for 2 days before excising the infarcted (white) tissue. The weight of infarcted tissue was expressed as a percentage of the total ventricle or the area at risk.

Western Blot Analysis
Anesthetic rats were perfused with saline and the hearts were prepared for Western blot analysis. Heart tissue was homogenized in Laemmli lysis buffer containing protease inhibitors (10 μl/0.2 g tissue weight, Sigma, USA). Protein concentrations in each sample solution were determined using a protein assay kit (BCA kit; Pierce, Rockford, IL, USA) and the samples were stored at –80°C until use. Aliquots containing 120 μg of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010; Millipore Corp, Bedford, MA, USA). Western blot analysis of NOS protein was performed as previously described. 

Table 1. Effect of Luteolin on Arrhythmias Induced by Coronary Ligation (30 min) in Anesthetized Rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>VT Incidence (%)</th>
<th>Duration (s)</th>
<th>VF Incidence (%)</th>
<th>Duration (s)</th>
<th>Mortality (%)</th>
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<td>Sham</td>
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<tr>
<td>Vehicle</td>
<td>4</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Luteolin 1 μg/kg</td>
<td>4</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Operated (ligated)</td>
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<tr>
<td>Vehicle</td>
<td>15</td>
<td>87</td>
<td>42.9±12.8</td>
<td>67</td>
<td>81.0±23.7</td>
<td>53</td>
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<tr>
<td>Luteolin 0.01 μg/kg</td>
<td>8</td>
<td>50</td>
<td>23.0±13.5</td>
<td>38</td>
<td>33.6±20.1</td>
<td>25</td>
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<tr>
<td>Luteolin 0.1 μg/kg</td>
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<td>50</td>
<td>7.7±4.1</td>
<td>25</td>
<td>12.7±12.3</td>
<td>13</td>
</tr>
<tr>
<td>Luteolin 1 μg/kg</td>
<td>8</td>
<td>25*</td>
<td>5.4±4.0*</td>
<td>25</td>
<td>3.1±2.4*</td>
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<tr>
<td>Luteolin 10 μg/kg</td>
<td>8</td>
<td>25*</td>
<td>2.7±2.5*</td>
<td>13*</td>
<td>3.7±3.7*</td>
<td>0*</td>
</tr>
</tbody>
</table>

Vehicle is 0.01% DMSO in normal saline; n = number of experiments; values for duration of VT and VF are shown as the mean ± SEM.

*Statistical difference at the level of P<0.05 as compared with vehicle.

VT, ventricular tachycardia; VF, ventricular fibrillation.

Table 2. Effect of Luteolin on Arrhythmias Induced by Coronary Reperfusion (30 min) in Anesthetized Rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>VT Incidence (%)</th>
<th>Duration (s)</th>
<th>VF Incidence (%)</th>
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<tbody>
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<td>Sham</td>
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<tr>
<td>Vehicle</td>
<td>4</td>
<td>–</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Luteolin 1 μg/kg</td>
<td>4</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Operated (reperfused)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>15</td>
<td>67</td>
<td>15.7±5.2</td>
<td>67</td>
<td>77.8±18.5</td>
<td>53</td>
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<tr>
<td>Luteolin 0.01 μg/kg</td>
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<td>50</td>
<td>3.7±2.4</td>
<td>38</td>
<td>38.3±34.2</td>
<td>25</td>
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<tr>
<td>Luteolin 0.1 μg/kg</td>
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<td>50</td>
<td>2.0±0.9*</td>
<td>13*</td>
<td>0.7±0.7*</td>
<td>0*</td>
</tr>
<tr>
<td>Luteolin 1 μg/kg</td>
<td>8</td>
<td>13*</td>
<td>0.6±0.6*</td>
<td>0*</td>
<td>0.0±0.0*</td>
<td>0*</td>
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Vehicle is 0.01% DMSO in normal saline.

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VT, ventricular tachycardia; VF, ventricular fibrillation.

Western Blot Analysis
Anesthetic rats were perfused with saline and the hearts were prepared for Western blot analysis. Heart tissue was homogenized in Laemmli lysis buffer containing protease inhibitors (10μl/0.2g tissue weight, Sigma, USA). Protein concentrations in each sample solution were determined using a protein assay kit (BCA kit; Pierce, Rockford, IL, USA) and the samples were stored at –80°C until use. Aliquots containing 120μg of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010; Millipore Corp, Bedford, MA, USA). Western blot analysis of NOS protein was performed as previously described. Protein bands were transferred onto a PVDF membrane (IPVH00010; Millipore Corp, Bedford, MA) and probed for inducible NOS (iNOS) (1:1,000 [catalog no. N32020; Transduction Laboratories, Lexington, KY, USA]), neuronal NOS (nNOS) (1:1,000 [catalog no. N41520; Transduction Laboratories]), endothelial NOS (eNOS) (1:1,000 [catalog no. N30020; Transduction Laboratories]) and 1:2000 actin (sc-1616 Santa Cruz Biotechnology, Santa Cruz, CA, USA) by incubation in the primary antibody, followed by a horseradish peroxidase–conjugated secondary antibody 1:1,000 (catalog no. M15345 for NOS; Transduction Laboratories and catalog no. 7074 for actin; Cell Signaling Technology, Inc, Beverly, MA, USA). Blots were visualized using the western lightning chemiluminescence reagent (Perkin Elmer Life Science, Inc, Boston, MA, USA) according to the manufacturer’s directions, and then exposed to X-ray film.

Detection of mRNA Expression
The levels of nNOS, eNOS and iNOS mRNA were detected in the occluded zone of the heart by reverse transcription polymerase chain reaction (RT-PCR), as previously described. Total RNA was extracted from the heart tissue with RNase Maxi kits (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was then performed with 5μg of total RNA, the oligo(dT) primer (BRL), and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. RT-PCR was carried out in O’ in 1 DNA polymerase solution at 50°C for 60 min, followed by enzyme inactivation at 72°C for 15 min. The primer sequences were as follows:

nNOS forward primer: 5’-TTCCGAAGCTTCTGGCAA-3’, nNOS reverse primer: 5’-AGATC-
1. Amplification procedure consisted of initial denaturation at 95°C for 5 min, followed by cycle parameters of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, with controlled extension at 72°C for 1 min, for 35 cycles. The amplified products were separated by gel electrophoresis in 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. Each set of PCRs included control samples run without RNA or in which the RT step was omitted. The RT-PCR procedure was highly reproducible under the present experimental conditions.

Assessment of Lipid Peroxidation
The malondialdehyde (MDA) content of heart tissue homogenates was measured with a commercially available assay kit (Bioxytech MDA 586; Oxis Research, Portland, OR, USA). Colorimetric analysis was performed at 586 nm, and MDA production was read from a standard curve and corrected for tissue protein content (nmol/mg protein). Bradford’s method was used to assess protein concentration.

Drug Administration
Luteolin was purchased from the Sigma Chemical Company (St Louis, MO, USA) and fresh solution was prepared before administration. Luteolin (0.01, 0.1, 1 or 10 μg/kg) or vehicle (dimethyl sulfoxide –0.9% NaCl, 1:10; v/v) was infused via a jugular vein 15 min before coronary artery occlusion. Rats injected with vehicle were used as controls. At the given concentration, vehicle had no effects on ischemia- or reperfusion-induced arrhythmia and MI. Animals were randomly allocated to either drug treatment or vehicle administration.

Statistical Analysis
Data are expressed as mean±standard error of mean (SEM). Between-group differences in BP, HR, duration of VT and VF, infarct size, plasma LDH and NO levels were assessed by analysis of variance (ANOVA) followed by the Newman-Keuls test. The difference in the percentage incidence of VT, VF, and mortality was analyzed with a χ² test. P<0.05 was considered to be statistically significant.

Results

Hemodynamic Changes During Coronary Artery Occlusion
Jugular vein injection of luteolin did not change mean arterial BP or HR in rats subjected to myocardial IR injury. No significant differences were recorded between the vehicle- and luteolin-treated rats (data not shown).

Myocardial Ischemia-Induced Rhythm Disturbances
The effects of luteolin on coronary ligation-elicited arrhythmias in anesthetized rats are shown in Table 1. In the vehicle-treated group, severe ventricular arrhythmias occurred at 6–7 min and peaked at 8–12 min, and had normally subsided within approximately 15 min, after coronary occlusion. Among the 15 rats in the vehicle-treated group, 13 animals (87%) exhibited VF (81.0±23.7 s in duration) and 10 animals (67%) exhibited VT (42.9±12.8 s in duration) and VF (23.7±2.5 s; VF: 3.7±3 s). The mortality rate was significantly decreased from 53% to 0% in rats treated with 10 μg/kg luteolin.

Myocardial Reperfusion-Induced Rhythm Disturbances
The effects of luteolin on myocardial reperfusion-elicited arrhythmias in anesthetized rats are shown in Table 2. The severity of reperfusion-induced arrhythmias was critically dependent on the duration of the preceding period of ischemia. In this study, we selected a 5-min period of ischemia followed by a 30-min period of reperfusion in order to produce

Table 1: Effects of luteolin on myocardial reperfusion-induced arrhythmias in anesthetized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>VT (% of rats)</th>
<th>Mortality (% of rats)</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>87%</td>
<td>53%</td>
</tr>
<tr>
<td>Luteolin</td>
<td></td>
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<tr>
<td>0.01 μg/kg</td>
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<td>10 μg/kg</td>
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</table>

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<table>
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<tr>
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<th>VF (% of rats)</th>
<th>Mortality (% of rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>67%</td>
<td>37%</td>
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<tr>
<td>Luteolin</td>
<td></td>
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<tr>
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<td>10 μg/kg</td>
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</table>

Figure 1. Effects of luteolin on plasma lactate dehydrogenase (LDH) activity at the end of the myocardial ischemia (A) and reperfusion (B) phases. Each experimental group included 6 rats. Results are expressed as mean±SEM. Statistical analysis was performed by 1-way ANOVA followed by unpaired Student’s t-test (*P<0.05 vs. vehicle). Note that luteolin dose-dependently decreased plasma LDH activity in both ischemia and reperfusion treated rats.

TAAGGGCGGTGTGTCACTTC-3’, iNOS forward primer: 5′-TCACGACACCTCTACCACAA-3’, iNOS reverse primer: 5′-CCATCTCCCTGCCCCATCTTC-3’, eNOS forward primer: 5′-TGGCGAGCATCACCTACGA-3’, eNOS reverse primer: 5′-TCCCAGACATCAATACCT-3’, β-actin forward primer: 5′-CCAGAGCAAGAGGCATCTGC-3’, β-actin reverse primer: 5′-GCCGATGATGAGCCTGACG-3′.

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Myocardial Reperfusion-Induced Rhythm Disturbances
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maximal rhythm disturbance. During myocardial reperfusion injury, compared with vehicle-treated rats, the groups of rats administered 0.1 µg/kg and 1 µg/kg of luteolin had significantly lower durations of VT (15.7±5.2 s vs. 2.0±0.9 s and 0.6±0.6 s, respectively; P<0.05) and VF (77.8±18.5 s vs. 0.7±0.7 s and 0.0±0.0 s, respectively; P<0.05), but the incidence of both VT (13% vs. 67%, P<0.05) and VF (0% vs. 67%, P<0.05) was significantly reduced only by the 1 µg/kg luteolin dose. The mortality rate was lowered from 53% to 0% by 1 µg/kg luteolin.

Myocardial Injury

Cellular damage was examined by measuring LDH leakage into plasma at the end of the myocardial IR injury phase. The effects of luteolin on the changes in LDH activity in plasma during IR injury are shown in Figure 1. Low LDH activity in the plasma was recorded in the sham-operated animals (52.8±13.9 U/L; n=6). However, after myocardial IR injury, there was a large increase in LDH levels from baseline in the plasma of rats given vehicle (from 124.5±18.1 to 167.8±20.4 U/L; n=6). In contrast, the administration of luteolin dose-dependently reduced LDH release.

Plasma NO Concentration

The effects of luteolin on NO concentrations are shown in Figure 2. NO release was measured by the presence of NO and NO- in plasma. In sham-operated rats, plasma NO was 4.7±1.10 µmO/L (n=6). In the operated animals without luteolin treatment, the plasma NO concentration was 7.31±1.00 µmO/L during myocardial ischemia (n=6) and 7.99±1.03 µmO/L during reperfusion (n=6). Administration of luteolin decreased NO release from myocardial cells in a dose-dependent manner during both the ischemia and reperfusion phases.

Myocardial Infarct Size

The effects of luteolin on myocardial infarct size are shown in Table 3. There was no significant difference in the size of the area at risk between the vehicle- (68.3±2.9%) and luteolin- (69.7±0.9%) treated groups, which indicated that in each group, a similar amount of tissue was at risk from occlusion of the left coronary artery. In the vehicle-treated group, the infarct size was 18.4±1.5% of the area at risk. If 10 µg/kg luteolin was administered prior to occlusion, the infarct size was significantly reduced to 10.0±1.2% of the area at risk.

NOS Protein Expression

Levels of iNOS, eNOS, and nNOS proteins were similar in the non-occluded and occluded zones in the sham-operated rats. As shown in Figure 3, the density of NOS protein expression in the same samples was normalized with β-actin. Cardiac ischemia for 1 h and reperfusion for 3 h induced iNOS expression, whereas administration of luteolin 10 µg/kg prior to the operation significantly suppressed iNOS induction in the occluded zone. In contrast, levels of eNOS and nNOS protein expression were not significantly different between non-occluded and occluded heart tissue after IR injury, with or without luteolin treatment.

NOS mRNA Expression

Luteolin at the dose of 10 µg/kg reduced the iNOS mRNA signal in the occluded zone compared with the vehicle-treated group. However, eNOS and nNOS mRNA expressions in the non-occluded and occluded zones were not significantly different between the luteolin-treated group and the vehicle-treated group (Figure 4).

**Table 3. Weight and Size of Area at Risk (n=6)**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Luteolin (10 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV weight (g)</td>
<td>0.70±0.02</td>
<td>0.70±0.04</td>
</tr>
<tr>
<td>Area at risk (g)</td>
<td>0.50±0.03</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>Area at risk/LV (%)</td>
<td>68.3±2.9</td>
<td>69.9±0.9</td>
</tr>
<tr>
<td>Infarct size (g)</td>
<td>0.10±0.01</td>
<td>0.05±0.01*</td>
</tr>
<tr>
<td>Infarct size/LV (%)</td>
<td>11.8±1.2</td>
<td>7.0±0.9*</td>
</tr>
<tr>
<td>Risk zone infarcted (%)</td>
<td>18.6±1.5</td>
<td>10.0±1.2*</td>
</tr>
</tbody>
</table>

Data are mean±SEM. *P<0.05 compared with control group. LV, left ventricle.
MDA Levels
In vehicle-treated rats, after 1 h of myocardial ischemia and 3 h of reperfusion, MDA levels in heart tissue were significantly elevated compared with sham-operated rats (336.4±12.2 vs. 294.2±2.7 nmol·L⁻¹·mg⁻¹) (P<0.05). In the luteolin-treated group, MDA levels were significantly attenuated (226.6±18.7 nmol·L⁻¹·mg⁻¹; P<0.05) compared with vehicle-treated rats (Figure 5).

Discussion
In this study, we showed that administration of luteolin at 1 or 10 μg/kg significantly suppressed the incidence and duration of VT and VF and completely prevented mortality during myocardial IR injury. Our results indicate that luteolin has cardioprotective effects, consistent with our finding that pretreatment with luteolin decreased carotid blood LDH levels, which serve as an indicator of cellular damage, during the same period of IR. In addition, in animals subjected to 1 h of coronary artery occlusion and 3 h of reperfusion, the cardiac infarct zone was reduced by pretreatment with luteolin.

Our results also showed that plasma NO concentrations after myocardial IR injury were significantly decreased in a dose-dependent manner in the luteolin-treated groups compared with vehicle-treated rats. NO is a small, gaseous biological active messenger with a broad range of physiological and pathological actions. NO has vasodilative effects, antiplatelet activity and antiinflammatory activity, which are all beneficial for cardiac-related improvement after myocardial IR injury. However, the reputed cardiotoxic or cardio-
protective role of NO during myocardial IR injury generates considerable debate. NO can inhibit mitochondria function and break single-stranded DNA. In addition, NO and superoxide radicals can rapidly combine to form a strong reactive metabolite, peroxynitrite, which is a potent oxidant that can potentially cause membrane lipid peroxidation and lead to myocardial dysfunction.\textsuperscript{35,36} During the myocardial IR, luteolin suppression of NO production might prevent NO from interacting with superoxide radicals, thereby preventing free radical injury. The family of NOS enzymes comprises the constitutive nNOS, eNOS and iNOS enzymes. Beneficial effects of eNOS include vasodilation, inhibition of platelet aggregation and polymorphonuclear neutrophil adhesion, whereas nNOS and iNOS appear to be deleterious.\textsuperscript{37,38} Recently, Marieke et al showed that luteolin inhibited NO production and reduced the expression of iNOS in lipopolysaccharide-stimulated NR8383 macrophages.\textsuperscript{39} In our study, we found that iNOS protein was induced in rat heart tissue after myocardial IR injury and pretreatment with luteolin suppressed iNOS protein expression under these conditions. However, there was no significant difference in nNOS and eNOS protein expressions between the luteolin-treated group and the vehicle-treated group. Further investigations showed a similar result for mRNA expression. Luteolin downregulated iNOS mRNA expression, without influencing nNOS or eNOS mRNA expression. This finding suggests that the effect of luteolin on iNOS expression was through transcription. However, iNOS mRNA expression increased in both the non-occluded and occluded zones, which does not correlate with the induction of protein levels seen only in the occluded zones according to Western blot analysis. Our results suggest that iNOS mRNA is expressed in both the non-occluded and occluded zones, but that the myocardium expresses iNOS transcription only after IR injury.

Investigations support the contention that ROS play an important role in the pathophysiology of myocardial IR injury.\textsuperscript{42,43} The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins leads to metabolic, electrophysiological, and functional alterations of the myocardium, which may induce potentially lethal ventricular arrhythmia and myocardial necrosis.\textsuperscript{8,44} Luteolin is believed to be an antioxidant or a free radical scavenger in the biological system.\textsuperscript{21–23} In support of this contention, we found that levels of MDA, a lipid peroxidation product regarded as a presumptive marker for oxidative stress, were decreased in patients with myocardial ischemia/reperfusion injury. A clinical view on a complex pathophysiological process. Int J Cardiol 2005; 100: 179–190.


