行政院國家科學委員會專題研究計畫 成果報告

探討 Luteolin 之心臟保護機制
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 97-2320-B-040-003-
執 行 期 間 ：97 年 08 月 01 日至 98 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系藥理學科

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處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 98 年 09 月 04 日

目錄：

報告內容：

前言：

In our previously **grand NSC 95-2320-B-040-027** from the National Science Council and Academic Sinica, for estimate myocardial ischemia or ischemia-reperfusion (I/R) injury, we ligated left coronary artery 30 min or 5min ischemia following 30 min reperfusion to investigate the antiarrhythmic effects of luteolin in the rats under myocardial ischemia and I/R injury. We found that luteolin is a potent cardioprotective agent with antiarrhythmic effect in myocardial ischemia and I/R injury rats. During the same period, pretreatment with luteolin also decreased (lactate dehydrogenase) LDH levels, an indicator of cell damage, and nitric oxide (NO) production in the carotid blood.

研究目的：

There is no literature to consider luteolin for the possible use as a therapeutic drug in treating the acute scenarios on myocardial ischemia and reperfusion injury induced myocardial infarction. Therefore, in the present study, we evaluate the cardioprotective effect of luteolin on myocardial ischemia and reperfusion injury in anesthetized rats subjected to transient coronary artery occlusion and reperfusion. Animals were pretreated with or without luteolin before coronary artery ligation and the severity of myocardial ischemia and reperfusion induced infarct size were compared. We also test the lactate dehydrogenase (LDH) levels in the carotid blood to correlate cellular damage with the myocardial ischemia and reperfusion lesions treated by luteolin. In addition, the nitric oxide (NO) production in plasma and the expression of protein and mRNA of inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS) were examined

文獻探討：

Evidences from studies on the myocardium suggest that reactive oxygen species, including superoxide radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, contribute to the pathophysiology of myocardial ischemia and reperfusion injury.[1,2] The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins induce myocardial cell damage, leading to depressed cardiac function and irreversible tissue injury. Myocardial ischemia and reperfusion injury will induce potentially lethal ventricular arrhythmia resulting in circulation collapse and end up in sudden death.[3] Therefore, effective inhibition of reactive oxygen species production or elimination of oxygen-derived free radicals become important strategy for the treatment of the ventricular arrhythmia and myocardial infarction caused by myocardial ischemia or reperfusion injury.[4,5]

Luteolin is one of the most wildly presented flavonoids in many kinds of fruits and vegetables.[6,7] There are several literatures had been reported that luteolin possess antineoplastic,[8,9] antihepatotoxic, antiallergic, antiosteoporotic,[10] antidiabetic activity,[11] and anti-inflammatory activities.[12] Recently, luteolin also has been shown to protect DNA against free radicals injury in human melanoma HMB-2 cells.[13] The antioxidant activity of luteolin may have beneficial effect on the model of myocardial ischemia and reperfusion injury. Luteolin also has been shown to significantly enhance left ventricular pressure and the global and relative coronary flow in Langendorff rabbit hearts subjected to repetitive myocardial ischemia.[14]

研究方法：

Animals. The investigation conforms with 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). Male Sprague-Dawley rats (National Lab. Animal Breeding and Research Center, Taipei, Taiwan) weighting 250~300 g were used. These animals were housed in a room with controlled temperature (24±1°C) and humidity (55±5%) under a 12:12 h light-dark cycle. They were allowed free access to food and water.

Surgical procedure. Rats undergoing myocardial ischemia and reperfusion injury were induced by a temporary occlusion of the left main coronary artery in procedures as described previously.[15] Briefly, Male sprangue-Dawley 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 and arterial blood pressure by a Statham P23 XL transducer and displayed on a Gould RS-3400 physiological recorder (Gould, Cleveland, OH, USA). A standard lead-1 electrocardiogram (ECG) was also recorded by attaching silver electrodes to extremities of animals.

After tracheotomy, the animals were ventilated with room air by a respirator for small rodents (Model 131, NEMI, U.S.A.) with a stroke volume of 15 ml/kg body weight and at a rate of 60 strokes/min to maintain normal P_{O_2}, Pc_{O_2} and pH parameters (blood gas analyzer, GEM-5300 I.L. CO, USA). The chest was opened by a left thoracotomy, followed by sectioning the fourth and fifth ribs, approximately 2 mm to the left of the sternum. The heart was quickly expressed out of the thoracic cavity, 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 then was occluded by tightening the ligature and reperfusion was achieved by releasing the tension applying 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 the silk passing around the left coronary artery was not tied (sham groups).[16]

Estimation of myocardial infarct size. Only rats that survived one hour coronary ischemia and three hours reperfusion were included for evaluation of infarct zone. Occluded zone and infarct zone in rat heart were determined following the procedures previously described by Hung et al.[18] At the end of experiment, the coronary artery was reoccluded and 2.0 ml 3% methyl blue was injected intravenously to denote the area at risk. With this technique, the previously non-ischemic area appears blue whereas the area at risk remains unstained. The latter region was cut out, weighed and expressed occluded zone as 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 (TTC, 1%; Sigma, USA) in normal saline) at 37°C in the dark for 40 min. Sections were then placed in 10% formaldehyde in saline for 2 days before infarct (white) tissue was excised. The weight of infarct tissue was expressed as percentage of the total ventricle or the area at risk.

Drug administration. Luteolin was purchased from Sigma Chemical Company (St. Louis, Mo. USA) and luteolin solution was fresh prepared before administration. Luteolin (0.01, 0.1, 1 or 10 μg/kg) or vehicle (dimethyl sulfoxide-0.9% NaCl, $1{:}10^4$; v/v) were infused via a jugular vein 15 min before coronary artery occlusion. Rats injected with vehicle were used as control. No effect of vehicle on ischemia and reperfusion induced arrhythmia and infarction at such concentration. Animals were randomly allocated to each drug treatment and vehicle group.

Plasma NO Analysis. Arterial blood samples were drawn from the carotid catheter at the end of ischemia or reperfusion period. The deproteinized plasma samples were frozen and kept until analysis. For measurement of NO we employed the NO/ozone chemiluminescence technique (280 NOA^{TM}, Sievers Instruments, Boulder, CO 80303, USA).[20] The detection of plasma NO level is based on its reaction with ozone, which leads to the emission of red light. The photons from this reaction are detected and transformed to an electrical signal by a photomultiplier tube (PMT). Due to the use of filters in front of the PMT, NO/O3 chemiluminescence recorded with the Sievers NOA 280^{TM} is highly specific for NO. The current from the PMT is A/D converted and fed into a PC running the Asyst software (Sievers NO Analysis Liquid Program, USA). The amount of light produced by NO/O3 chemiluminescence is proportional to the amount of NO sampled. Hence, the calculated area

under the curve of the PMT current for each determination is proportional to the amount of NO. This was verified before each experiment by standard curves (1, 5, 10, 20, 40, 100 μmol/L) which were produced using freshly prepared solutions of sodium nitrite in distilled water, which was reduced to NO in an equimolar manner by the reducing agent. We chose to measure the level of nitrate and nitrite in blood sample, by using a reaction vessel containing a reducing system (Vanadium (III) dissolved in 1 M HCl), to which the sample was injected and NO was generated from nitrite or nitrate in an equimolar manner. A continuous stream of Helium (99.999%) purged the resultant NO from the reaction vessel to the chemiluminescence chamber.

Western blot analysis. 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, St. Louis, MO). Protein concentrations in each sample solution were determined using a protein assay kit (BCA kit; Pierce, Rockford, IL) 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 to polyvinylidene difluoride membrane (IPVH00010; Millipore Corp., Bedford, MA). Western blot analysis of NOS protein was performed as previously described.[18] Protein bands were transferred to polyvinylidene difluoride membrane (IPVH00010; Millipore Corp., Bedford, MA) and probed for iNOS (1:1000 [catalog no. N32020; Transduction Laboratories, Lexington, KY]), nNOS (1:1000 [catalog no. N41520; Transduction Laboratories, Lexington, KY]), eNOS (1:1000 [catalog no. N30020; Transduction Laboratories, Lexington, KY]) and 1:2000 actin (sc-1616 Santa Cruz Biotechnology, Santa Cruz, CA) by incubating in the primary antibody, followed by a horseradish peroxidase–conjugated secondary antibody 1:1000 (catalog no M15345 fon NOS; Transduction Laboratories, Lexington, KY and catalog no 7074 for actin; Cell Signaling Technology, Inc., USA). Blots were visualized using the western lightning chemiluminescence reagent (PerkinElmer Life Science, Inc., Boston) according to the manufacturer's directions, and were exposed to x-ray film.

Reverse transcription polymerase chain reaction (RT-PCR). nNOS, eNOS and iNOS mRNA were detected in the occluded zone of the heart by RT-PCR, as previously described.[18] Total RNA was extracted from the heart tissue with RNase Maxi kits (Qiagen, Valencia, CA,USA). First strand cDNA synthesis was then performed with the use of 5 μg of total RNA, oligo (dT) primer (BRL), and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RT-PCR was carried out in O' in 1DNA 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'-TTCCGAAGCTTCTGGCAACAGCGACAATTT-3'.

nNOS reverse primer: 5'-AGATCTAAGGCGGTTGGTCACTTC-3'.

iNOS forward primer: 5’-TCACGACACCCTTCACCACAA-3’.
iNOS reverse primer: 5’-CCATCCTCCTGCCCACTTCCTC-3’.
eNOS forward primer: 5’-TGGGCAGCATCACCTACGA-3’.
eNOS reverse primer: 5’-TCCCGAGCATCAAATACCT-3’.
β-actin forward primer: 5’-CCAGAGCAAGAGAGGCATCCTG-3’.
β-actin reverse primer: 5’-GCCGATAGTGATGACCTGACCGT-3’.

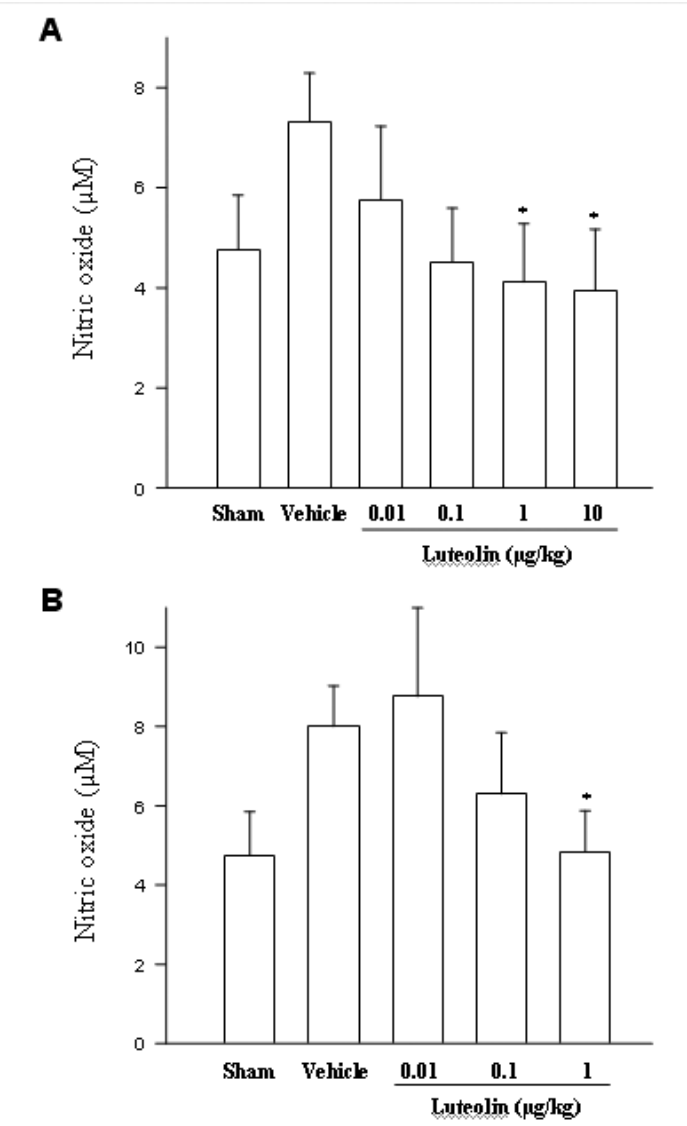
The amplification procedure consisted of initial denaturation at 95℃ for 5 min, following cycle parameters of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 35 cycles. The amplified products were separated gel electrophoresis in 1.5% agarose gel containing 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.

Statistics. Data were expressed as mean ± standard error of mean (SEM). The difference of blood pressure, heart rate, duration of VT and VF, and infarct size, plasma LDH and NO levels between vehicle and drug treatment groups were carried out by using analysis of variance (ANOVA) followed by Newman-Keuls test. The difference in the percentage incidence of VT, VF and mortality were analyzed with a χ^2 test. $P < 0.05$ was considered to be statistically significant.

結果：

Hemodynamic changes during coronary artery occlusion. Jugular vein injection of luteolin did not change the mean arterial pressure nor the heart rate in rats subjected to myocardial ischemia or reperfusion injury. No significant difference was seen among vehicle and luteolin treated groups (data not show).

Plasma NO. The effects of luteolin on NO contents are shown in Fig 1. NO release was measured by the presence of nitrite (NO_2^-) and nitrate (NO_3^-) in plasma. In sham-operated rats, plasma NO was 4.76 ± 1.10 μmol/L (n=6). In the operated animals without luteolin treatment, the NO content in plasma of rats subjected to myocardial ischemia and reperfusion were 7.31 ± 1.00 μmol/L (n=6) and 7.99 ± 1.03 μmol/L (n=6), respectively. Administration of luteolin decreased the NO release in a dose-dependent manner during myocardial ischemia and reperfusion period.



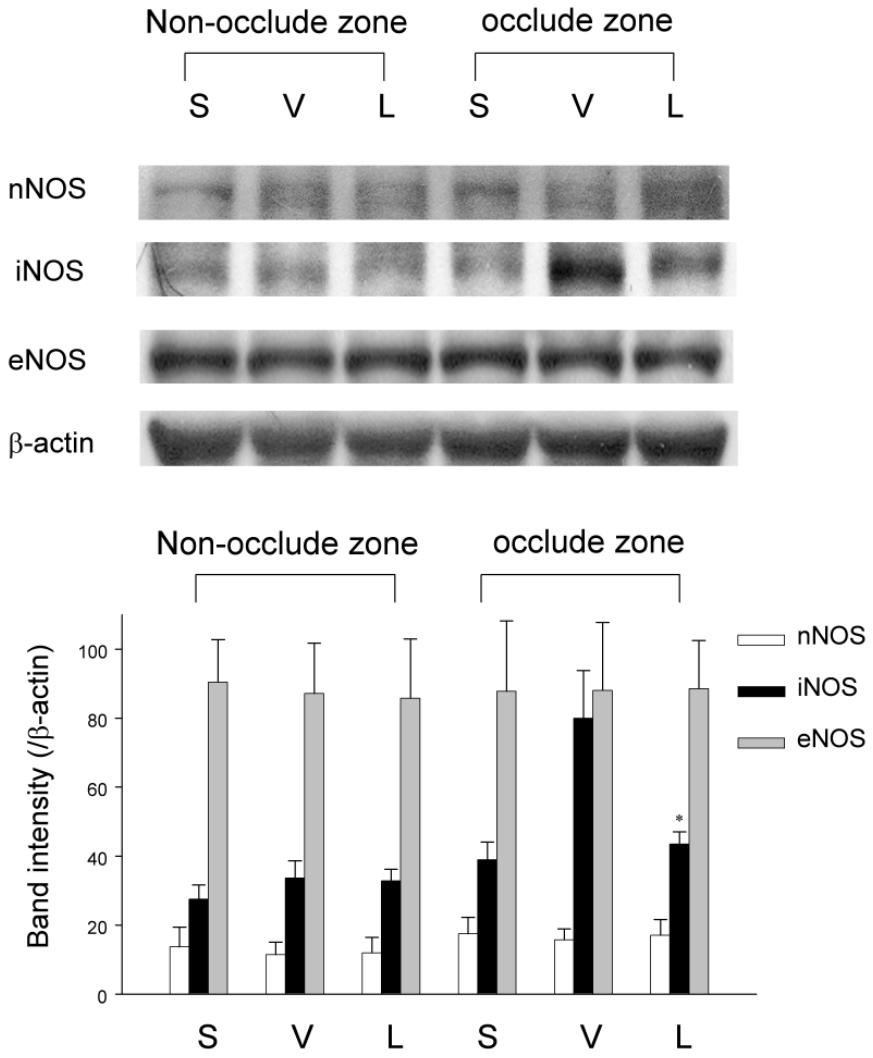
Myocardial infarct size. The effects of luteolin on myocardial infarct size are shown in Table I. There was no significant difference in the size of area at risk between vehicle (68.3 ± 2.9%) and luteolin (69.9 ± 0.9%) treated group. This indicated that a similar amount of tissue was imperiled by occlusion of the left coronary artery in each group. In the vehicle group, the infarct size was 18.6 ± 1.5% of area at risk. If 10 μg/kg luteolin was preadministrated, the infarct size in rats significantly reduced to 10.0 ± 1.2% of area at risk.

Table I. Weights and Size of Area at Risk (n=6)

	Vehicle	Luteolin (10 ug/kg)
LV weight (g)	0.70±0.02	0.70±0.04
Area at risk (g)	0.50±0.03	0.49±0.03
Area at risk/LV (%)	68.3±2.9	69.9±0.9
Inarct size (g)	0.10±0.01	0.05±0.01*
Infarct size/LV (%)	11.8±1.2	7.0±0.9*
Risk zone infarcted (%)	18.6±1.5	10.0±1.2*

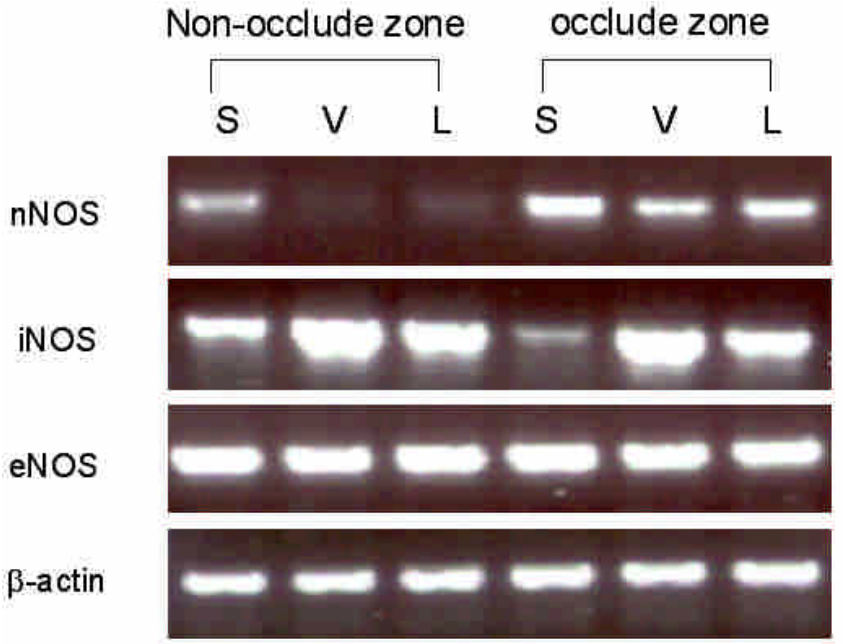
Data are mean ± SEM.
LV = left ventricular;
* P<0.05 compared with control group.

NOS protein expression. It was noticed that the levels of iNOS, eNOS, and nNOS proteins in either non-occluded zone or occluded zone of sham-operated rats were similar. As shown in Fig. 2, the density of NOS protein expression was normalized with β-actin from the same samples. Cardiac one hour ischemia and 3 hours reperfusion induced iNOS expression and administration 10 μg/kg luteolin prior to operation significantly suppressed iNOS induction in the occluded zone. In contrast, the protein level of eNOS and nNOS expression was not significantly difference with or without luteolin treatment after ischemia and reperfusion injury between non-occluded and occluded heart tissue. Each value represents the mean of 6 individual experiments.



NOS mRNA expression. Luteolin at the dose of 10 μg/kg reduced the iNOS mRNA signal in occluded zone as compared with the vehicle-treated group. However, there was not

affected the eNOS and nNOS mRNA expression in either non-occluded zone or occluded zone between luteolin-treated group and vehicle-treated group (Fig. 3). Each value represents the mean of 6 individual experiments.



討論：

Ischemia is characterized in part by low tissue oxygen tension. It is well documented that salvage of the ischemic myocardium is dependent upon timely reperfusion**,** it is likely that the very events critical for survival may, in fact, lead to further tissue injury. There are several evidences from studies on the myocardium suggest that reactive oxygen species (superoxide radical, hydrogen peroxide, hydroxyl radical, singlet oxygen) contribute to the pathophysiology of myocardial ischemia and reperfusion injury. These reactive oxygen species, which are formed within the myocardial ischemia and first moments of reperfusion period, are known to be cytotoxic to surrounding cells.[21, 22, 23] Therefore, Myocardial ischemia and reperfusion injury will induce ventricular arrhythmia resulting in circulation collapse and end up in sudden death.[24, 25] The strong evidence lies in the ability of free radicals scavengers to reduce the ventricular arrhythmia and to limit myocardium damage in experimental models caused by myocardial ischemia and reperfusion injury.[4, 26]

In the present study, we showed that luteolin significantly reduced the cardiac infarct size on rats subjected to myocardial ischemia and reperfusion injury. In addition, in anesthetized rats the administration of luteolin prior to coronary artery occlusion significantly reduced the mortality and suppressed the myocardial arrhythmias during myocardial ischemia and reperfusion injury. That was consistent with the finding of luteolin pretreatment also decreased LDH, an indicator of cellular damage, levels in the carotid blood during the same

period. Theses results indicated that luteolin exhibit the cardioprotective effect against myocardial ischemia and reperfusion injury.

Luteolin is one of the most wildly distributed flavonoids, a group of naturally occurring polyphenolic compound, which present in many kinds of fruits and vegetables.[6] Luteolin has a wide range of biological and pharmacological properties including antineoplastic activities,[27, 28] anti-inflammatory effect,[12] antiplatelet and vasodilatory activity,[7] and antioxidant effect.[29] The cardioprotective effect of luteolin may be due to the antioxidant activity in biological systems. Luteolin had been reported to inhibit xanthine oxidase activity, which has been implicated in oxidative injury to tissue by ischemia-reperfusion, at low concentrations (IC_{50} value is 0.96 μM).[30] In human melanoma HMB-2 cells, luteolin showed a concentration-dependent inhibitory activity toward DNA damage induced by H_2O_2.[13] The mass production of oxygen-derived free radicals during myocardial ischemia and reperfusion period may be arrested by antioxidant activity of luteolin. In addition, in isolated rabbit heart, luteolin also possess cardioprotective properties against repetitive myocardial ischemia injury by improvement of left ventricular pressure. Luteolin also enhanced the global and relative coronary flow and therefore might improve myocardial perfusion in the ischemic border area.[14] Recently, Marieke et al., showed that luteolin inhibited nitric oxide (NO) production and reduced the expression of inducible NO synthase (iNOS) in lipopolysaccharide stimulated NR8383 macrophages.[31] NO is an agent that can induce cell damage. NO can inhibit mitochondria function and break DNA single-strand. 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 leading to myocardial dysfunction.[32] In this study, we found that NO contents in plasma were significantly decreased with a dose-dependent manner in luteolin treated group compared with vehicle treated group in rat after myocardial ischemia and reperfusion injury. In addition, we showed that luteolin decreased NO production by downregulation of protein and mRNA expression of iNOS, while the expression of protein and mRNA of nNOS and eNOS was unchanged. During myocardial ischemia and reperfusion period, luteolin suppressed NO production might to prevent NO interaction with superoxide radicals, which then to avoid the injury of free radicals.

In conclusion, our study presents the first evidence that pretreatment with luteolin could effectively protect myocardium against myocardial ischemia and reperfusion induced cardiac injury. We wound speculate that the beneficial cardioprotective effect of luteolin may be corrected with it decrease the production of NO.

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計畫成果自評：

This study had finished and prepared for publish.