# 行政院國家科學委員會專題研究計畫　成果報告

# 一氧化氮在 resveratrol 保護局部大腦缺血傷害中所扮演的角色

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中文摘要及關鍵詞：

先前的研究，我們發現在局部大腦缺血傷害的大花鼠投與 resveratrol 可以減少梗塞體積，最近有很多文獻報導 resveratrol 藉由增加一氧化氮的機轉保護脊隨、腎臟和心臟對抗缺血以及再灌注傷害，本計畫研究一氧化氮在 resveratrol 保護大花鼠對抗局部大腦缺血傷害的神經保護作用機轉中所扮演的角色。實驗藉由將麻醉大花鼠的中大腦動脈結紮一小時而後再灌注二十四小時引起局部大腦缺血的傷害，resveratrol 在中大腦動脈結紮一小時後經由靜脈投與。

投與濃度 0.1 或是 1 μg/kg 的 resveratrol，在大花鼠遭受局部大腦缺血傷害，可以有意義的減少大花鼠血漿中 LDH 的濃度以及在大腦組織中 MDA 的量，相反的可以有意義的增加大花鼠血漿中一氧化氮（nitric oxide）的量。我們進一步測量各組間各種一氧化氮合成酵素(nitric oxide synthase, NOS)蛋白質表現量以及 mRNA 表現量之相關性，研究發現在治療投與 resveratrol 有意義的抑制 inducible nitric oxide synthase (iNOS)的蛋白質表現量以及 mRNA 表現量，卻會增加 endothelial nitric oxide synthase (eNOS) 蛋白質表現量以及 mRNA 表現量，而對於 neuronal nitric oxide synthase (nNOS)的蛋白質表現量以及 mRNA 表現量並沒有影響。預先投與非選擇性 NOS 抑制劑 L-NAME 可以拮抗 resveratrol 對抗大花鼠局部大腦缺血傷害的神經保護作用。綜合研究成果，我們認為在於大花鼠遭受局部大腦缺血傷害，治療投與 resveratrol 是很有效的神經保護劑，可以治療大花鼠遭受局部大腦缺血的傷害，這個研究證實一氧化但在 resveratrol 的神經保護作用中所扮演的重要角色。

關鍵字： Resveratrol；紅酒；中風；局部大腦缺血；Nitric oxide synthase (NOS)；梗塞體積；一氧化氮

英文摘要及關鍵詞：

In our previously study showed that resveratrol could suppress infarct volume and to exert neuroprotective effect on rats subjected to focal cerebral ischemia (FCI) injury. Recently, there are several literatures to report resveratrol protect spinal core, kidney, and heart from ischemia-reperfusion injury through upregulation of nitric oxide (NO). This study was designed to investigate the role of nitric oxide on the protective mechanisms of resveratrol on rats after FCI injury. The FCI injury was induced by the 1 hr middle cerebral artery (MCA) occlusion and 24 hr reperfusion in anesthetized Long-Evans rats. Resveratrol was intravenous injected after 1 hr MCA occlusion.

Treatment of resveratrol (0.1 and 1 μg/kg) decreased the lactate dehydrogenase (LDH) in plasma and malondialdehyde (MDA) in FCI injury brain tissue, whereas the level of NO in plasma was increased. In addition, resveratrol downregulated protein and mRNA expression of inducible nitric oxide synthase (iNOS), however, upregulated protein and mRNA expression of endothelial nitric oxide synthase (eNOS), while the expression of protein and mRNA of neuronal nitric oxide synthase (nNOS) was unchanged. Pretreatment with L-NAME, the nonselective NOS inhibitor, was completely blocked the decrease infarction effect of resveratrol. This study demonstrated the important role of NO in the neuroptotective effect of resveratrol in FCI injury.

**Key words**—Resveratrol; Red wine; Stroke; Focal cerebral ischemia; Infarct maturation; NO

# 目　　錄

報告內容：

前言：

Nitric oxide (NO), a cellular signaling molecule, is synthesized during the stoichiometric conversion of L-arginine to L-citrulline in the presence of oxygen and NADPH which is catalyzed by nitric oxide synthase (NOS).[1] There are three isoforms of NOS that are named by the tissue which they are first cloned. Neuronal NOS (nNOS; 157 kDa) and endothelial NOS (eNOS; 140 kDa) are constitutively expressed and are calcium dependent, whereas inducible NOS (iNOS; 135 kDa) is expressed after immunologic challenge and neuronal injury and is calcium dependent under most circumstance.[2] NO has a variety of physiological and pathological effects in biological systems.[3] Under normal physiological conditions, NO is an important endogenous vasodilator that can regulate cerebral blood flow and mediate vascular response to a diverse group of stimulation.[4] In addition, NO can inhibit platelet aggregation[5] and inhibit both platelet[6] and polymorphonuclear neutrophil (PMN)[7] adhesion to the vascular endothelium. Several lines of evidence have reported that NO donor or L-arginine exerts neuroprotective effect in stoke model. The findings indicate that NO donor, sodium nitroprusside, improves blood flow and reduces tissue damage after focal cerebral ischemia.[8] In both normotensive and hypertensive animals, L-arginine induces sustained cerebral blood flow increases in normal brain as well as in a marginally perfused brain region distal to middle cerebral artery (MCA) occlusion and decrease infarction volume after MCA occlusion.[9]

Resveratrol, a polyphenol phytoalexin (trans 3,4',5-trihydroxystilbene), is abundantly available in a wide variety of plant species. It is present in the skin and seeds of grapes and constitutes one of the major components of red wine.[10] Resveratrol has been reported to have a host of physiological effects including to prevent lipid peroxidation in human LDL,[11] to inhibit arachidonate acid metabolism,[12] to inhibit platelet activity.[13] Resveratrol was also found to stimulate nitric oxide (NO) production in endothelial cells to exert vasodilatory effect on blood vessels [14] and to interfere with the release of inflammatory mediators by activated human polymorphonuclear leukocytes (PMN) and down-regulates adhesion-dependent thrombogenic PMN functions.[15] Resveratrol is considered as a potential neuroprotective agent in treating acute scenarios in focal cerebral ischemia (FCI) injury. Our previously study showed that resveratrol could effectively suppress infarct size from the damaging effects of FCI.[16] Recently, resveratrol was found to protect spinal core,[17] kidney,[18] and heart [19] from ischemia-reperfusion injury through upregulation of NO.

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研究目的：

On the basis of these findings, the purpose of this experimental study was to determine the role of NO in the neuroprotective effect of resveratrol on rat subjected to FCI injury.

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研究方法：

**Animals**

The present investigation conforms to the Guide for the Car and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Long-Evans rats (National Lab. Animal Breeding and Research Center) weighing 250-320g were used throughout this study. 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**

Long-Evans rat was anesthetized with halothane (1% to 3.5% in a mixture of 70% $N_2O$ and 30% $O_2$) with the use of a mask. Body temperature was maintained during surgery at 37±0.5°C with a heating pad servo-controlled by a rectal probe. The right femoral artery was cannulated with PE-50 polyethylene catheters for continuous monitoring of heart rate and mean arterial blood pressure (MABP) by Statham P23 XL transducer and displayed on a Gould RS-3400 physiological Recorder (Gould, Cleveland, OH, USA) and blood sampling for analysis of blood gases by Blood Gas Analyzer (GEM-5300 I.L. CO, USA).

Focal ischemic infarcts were made in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA). Both common carotid arteries were exposed by midline anterior cervical incision. The animal was placed in a lateral position, and a skin incision was made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle was retracted, and a small (3-mm diameter) craniectomy was made at the junction of the zygoma and squamosal bone using a drill (Dremel Multipro+5395, Dremel com. USA) cooled with saline solution. Using a dissecting microscope (OPMI-1, ZISS, Germany), the dura was opened with fine forceps, and the right MCA was ligated with 10-0 monofilament nylon ties. Both common carotid arteries were then occluded by microaneurysm clips for 1 hr. After removing the clips, return of flow was visualized in the arteries.[20]

**Infarct volume analysis**

Twenty-four hours after cerebral infarction, animals were anesthetized and killed by rapid decapitation. Brain were removed, inspected visually for the anatomy of the MCA and for signs of hemorrhage or infection, immersed in cold saline solution for 10 minutes, and sectioned into standard coronal slices (each 2-mm thick) using a brain matrix slicer (JACOBOWITZ Systems, Zivic-Miller Laboratories INC, Allison park, USA). Slices were placed in the vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%; Sigma, USA) at 37°C in the dark for 30 minutes, followed by 10% formalin at room temperature overnight. The outline of right and left cerebral hemispheres as well as that of infarct tissue, clearly visualizable by a lack of TTC staining,[20] was outlined on the posterior surface of each slice using an image analyzer (color image scanner, EPSON GT-9000, connected to an image analysis system (AIS software, Imaging research INC, Canada) run on a personal computer, AMD K6-2 3D 400. Infarct volume was calculated as the sum of infarct area per slice multiplied by slice thickness. Both the surgeon and image analyzer

operator were blinded to the treatment given each animal.

**Plasma lactate dehydrogenase (LDH) and NO analysis**

Cellular damage was evaluated by measuring the LDH in plasma. Samples of arterial blood were drawn from the carotid catheter at the end after 1hr MCA occlusion and 24 hr reperfusion, collected in heparinized tubes. The blood was kept at 4°C until it was centrifuged at 2000 × g for 15 min. The plasma was recovered and aliquots were used for determination of LDH activity. LDH activity was measured spectrophotometrically, according to the method of Bergmeyer and Brent,[21] by following the rate of conversion of NADH to $NAD^+$, at 340 nm..

The deproteinized plasma samples were frozen and kept until analysis. For measurement of NO we employed the NO/ozone chemiluminescence technique (280 NOA™, Sievers Instruments, Boulder, CO 80303, USA).[22] 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, $NO/O_3$ chemiluminescence recorded with the Sievers NOA 280™ 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 $NO/O_3$ 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 nitrite or nitrate on 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 brains were prepared for Western blot analysis. Brains were quickly removed and homogenized in lysis buffer containing protease inhibitors. 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.[23]

**Reverse transcription polymerase chain reaction (RT-PCR)**

nNOS, eNOS and iNOS mRNA were detected in the occluded zone of the brain by RT-PCR, as previously described.[19] Total RNA was extracted from the brain 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 are shown in Table 1. The amplification procedure consisted of initial denaturation at 95°C 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.

**MDA assay**

MDA levels were measured by using the thiobarbituric acid method described by Okhawa et al.[24] Briefly, brain tissues were homogenized with 10 times (w/v) sodium phosphate buffer. The reagents of 1.5 ml 20% of acetic acid, 0.2 ml 8.1% of sodium dodecyl sulfate, 1.5 ml 0.8% of thiobarbituric acid was added to 0.1 ml tissue sample. The mixture was then heated at 100°C for 60 min. After cooling, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine was added and centrifuged. The organic layer was withdrawn and measured at 532 nm. MDA concentrations in the samples were calculated by a standard calibration curve by using 1,1,3,3-tetramethoxypropane prepared in the same manner. Each measurement was performed in duplicate. MDA concentration were expressed as nanomoles per gram tissue weight.

**Drug administration**

Resveratrol (Sigma, USA) was dissolved in 40% (v/v) propylene glycol to the desired concentrations in normal saline. Final concentration of propylene glycol in the infected resveratrol solution was $4\times10^{-3}$ % (v/v). At this concentration, propylene glycol had no effect on the infarct size of focal cerebral ischemia. In this study, resveratrol solution of 0.3 ml was administered at three different doses ($10^{-6}$, $10^{-7}$ and $10^{-8}$ g/kg) via intravenous injection when the common carotid arteries clips were removed. Rats injected with 0.3 ml vehicle were used as controls. Sham operated animals underwent all surgical procedure except MCA ligation. For examine which NOS was responsible for the neuroprotective effect of resveratrol in FCI injury, we administrated a nonselective NOS inhibitor (L-NAME), a specific nNOS inhibitor (7-nitroindazole), and a specific iNOS inhibitor (S-methylisothiourea sulfate) 15 minutes before MCA occlusion to examine their antagonistic effect with resveratrol. Animals were randomly allocated to each drug treatment and control groups.

**Statistics**

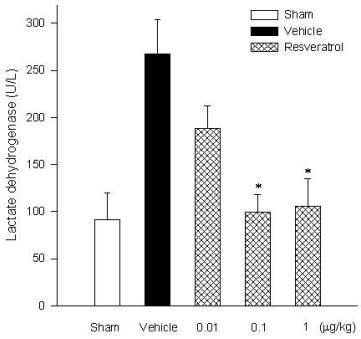
Data are expressed as mean ± standard error of mean (SEM). Statistical analysis of differences in Plasma LDH and NO levels, physiological measurements, infarct volume between vehicle and drug treated groups were carried by one-way analysis of variance (ANOVA) for

combined data and followed by unpaired, two-tailed Student's *t*-tests. $P < 0.05$ was considered to be statistically significant.

結果：

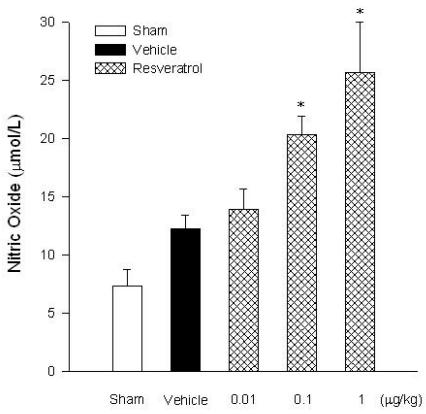
**Plasma LDH levels**

Cellular damage was evaluated by measuring the LDH level in plasma at the end period of focal cerebral ischemia. The effects of resveratrol on LDH activity were shown in Fig. 1. Low LDH activity was seen in sham-operated animals (91.5±28.7 U/L) before occlusion. In the operated animals without resveratrol treatment, large increase in the LDH activity was observed in the plasma (267.7±36.3 U/L). In contrast, administration of resveratrol attenuated of LDH release with a dose-dependent manner during 1 hr MCA occlusion and 24 hr reperfusion. Resveratrol at the dose of 1 μg/kg reduced the LDH activity to 105.4±29.3 U/L (n=7).



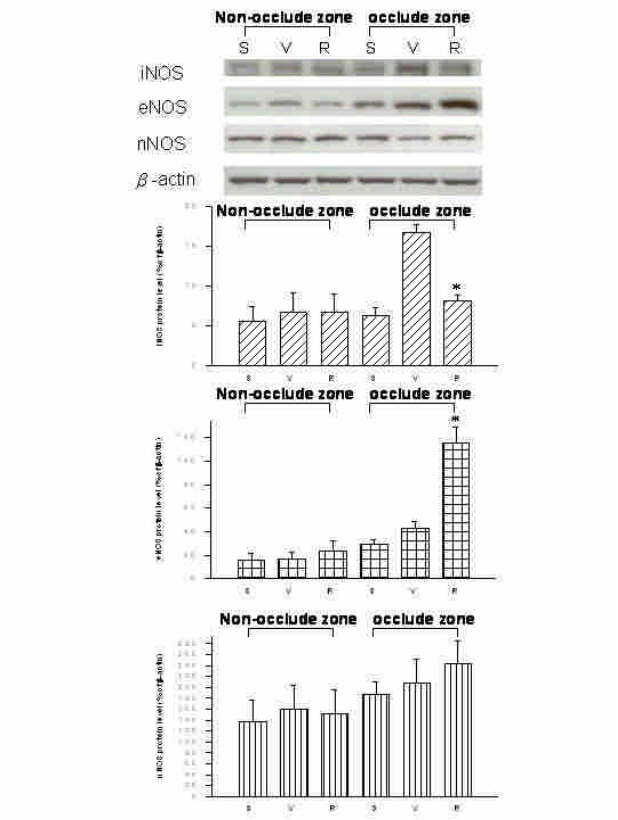

**Plasma NO**

The effects of resveratrol on NO contents were shown in Fig. 2. NO release was measured by the presence of nitrite ($NO_2^-$) and nitrate ($NO_3^-$) in plasma. The NO content in sham-operated rats was 7.35±1.41 μmol/L. In the operated animals without resveratrol treatment, the NO content in plasma of rats was 12.26±1.21 μmol/L. Administration of resveratrol increases the NO release with a dose-dependent manner during 1 hr MCA occlusion and 24 hr reperfusion. Resveratrol at the dose of 1 μg/kg, the plasma NO was increased to 25.70±4.28 μmol/L (n=7).



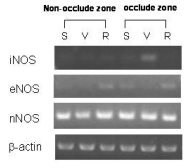
**Protein levels of NOS**

It is 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. 3, the density of NOS protein expression was normalized with β-actin from the same samples. Focal cerebral ischemia induced iNOS expression and administration resveratrol at the dose of 1 μg/kg significant suppressed iNOS induction in the occluded zone. In contrast, administration 1 μg/kg resveratrol significant increased the eNOS expression in the occluded zone as compared with vehicle-treated group. The protein level of nNOS expression was not significant difference with or without resveratrol treatment after focal cerebral ischemia between non-occluded and occluded brain tissue.



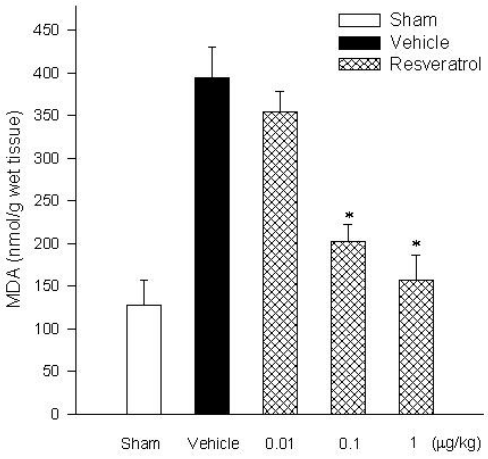

**NOS mRNA expression**

Resveratrol at the dose of 1 μg/kg reduced the iNOS mRNA signal in occluded zone as compared with the vehicle-treated group. However, administration 1 μg/kg resveratrol increased eNOS mRNA expression in occluded zone as compared with the vehicle-treated group. There was not affected the nNOS mRNA expression in either non-occluded zone or occluded zone between resveratrol-treated group and vehicle-treated group (Fig. 4).



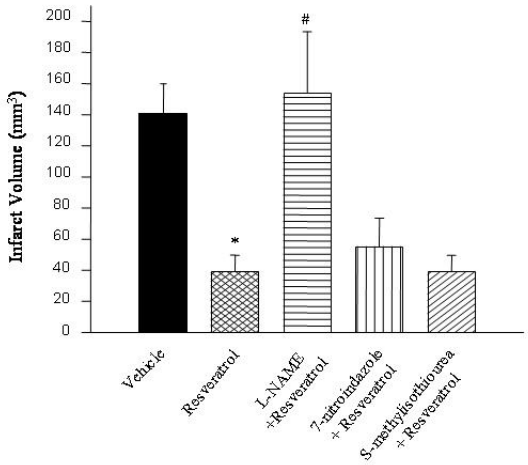
**MDA levels**

MDA levels in brain tissue after 1 hr of MCA occlusion and 24 hr reperfusion are shown in Table 5. Focal cerebral ischemia resulted in significant increases the levels of MDA in the group treated with vehicle (394.0±38.6 nmol/g wet tissue) as compared with sham group (127.8±26.8 nmol/g wet tissue). MDA levels in the resveratrol treated groups were decreased in a dose-dependent manner. Resveratrol at the dose of 1 μg/kg significantly reduced the MDA level to 157.3±31.3 nmol/g wet tissue (n=7).




**Infarct Volume**

The effects of resveratrol on infarct volume were shown in Fig. 6. The middle cerebral artery was occluded for 1 hr and then reperfused for 24 hrs, the infarct volume in the rats were 141.0±18.1 $mm^3$ (n=7). If 1 μg/kg resveratrol was treated immediately after release of the common carotid arteries clips, the infarct volume in rats significantly reduced to 39.1±10.6 $mm^3$ (n=7). The effect of resveratrol on decrease infarct volume was completely blocked by pretreatment with L-NAME (154.1±39.2 $mm^3$ (n=7)), but had no influence when pretreatment with 7-nitroindazole (55.2±18.3 $mm^3$ (n=7)) or S-methylisothiourea sulfate (38.8±10.8 $mm^3$ (n=7)).



討論：

In this study we demonstrated that treatment with resveratrol, at the doses of 0.1 and 1 μg/kg, significantly decreased LDH levels in the carotid blood on rats after FCI injury. The LDH activity in plasma was used as an indicator of cellular damage. The decreasing of LDH activity after resveratrol administration suggested that resveratrol might decrease the neuronal damages elicited after FCI injury. The result was consistent with the finding of our previously study which showed that resveratrol suppressed the total infarct volume in rats subjected to FCI injury.[16] The result indicated that resveratrol possessed robust neuroprotective effect against FCI in rats.

In addition, our results revealed that the NO contents in plasma were significantly increased with a dose-dependent manner in resveratrol-treated group as compared with vehicle-treated group on rats after FCI injury. NO is a small gaseous biologically active messenger with a wide rage of physiological and pathological actions.[3] NO possess vasodilatory effect,[4] anti-inflammatory activity,[7] and anti-platelet activity.[5,6] All these physiological effects of NO seem to be a beneficial role during FCI injury. However, it has been reported that the interaction between NO and superoxide radicals generates peroxynitrite, a potent free radical, which could induce lipid peroxidation of cellular membranes.[3] Current studies indicate that NO plays a dual role, may be protective or toxic effect on brain tissue under FCI injury, depend on its source. NO is produced by a family of isoenzymes termed of NOS.[4] The eNOS and nNOS are present under physiological conditions, whereas the iNOS is expressed in response to immunological stimulation. Among the three isoforms of NOS, eNOS produces NO with beneficial effects, such as vasodilative effect, anti-platelet aggregation effect, and inhibit PMN adhesion, whereas nNOS and iNOS produces NO with deleterious effects. Overproduction of NO from either nNOS or iNOS leads to neurodegeneration.[25] The receives further support from the evidence of the reduction brain edema and infarction volume on nNOS or iNOS knockout mice after FCI injury.[26,27] In this study we found that iNOS protein is induced in the rat brain tissue after FCI injury. Treatment with resveratrol suppressed iNOS protein expression in the same condition. Whereas, in rats treated with resveratrol significant increased eNOS protein expression in brain tissue after FCI injury as compared with vehicle-treated goup. But there was no affected on nNOS protein expression between resveratrol-treated group and vehicle-treated group. Future study showed the similar result on mRNA expression. Resveratrol downregulated iNOS mRNA expression, upregulated eNOS mRNA expression, and was no influence on nNOS mRNA expression. These results suggest that the effects of resveratrol on iNOS and eNOS expression were through transcription.

Resveratrol is a naturally polyphenolic compound that possesses antioxidant property. Indeed, in our study we found that administration of resveratrol decreased of MDA formation with a dose-dependent manner in brain tissue after FCI injury. That suggested resveratrol reduced the oxidative stress on rats due to FCI injury. A number of studies have demonstrated the role of free radicals on the injury of FCI.[28] The antioxidant activity of resveratrol may act through suppress superoxide radical to prevent the injury from the interaction of superoxide anion and NO and then to protect endothelium cell and leading to dose-dependent enhance NO production.. NO is known to possess vasodilatory activity,[4] inhibit platelet aggregation,[5] and inhibit platelet[6] and PMN[7]

adhesion to the vascular endothelium, which is beneficial for the improvement of FCI injury.

For investigate which NOS was responsible to the neuroprotective effect of resveratrol in rats after FCI injury, we administrated a nonselective NOS inhibitor (L-NAME), a specific nNOS inhibitor (7-nitroindazole), and a specific iNOS inhibitor (S-methylisothiourea sulfate) 15 minutes prior to MCA occlusion to examine their antagonistic effect with resveratrol. Our studies found that nNOS and iNOS inhibitors did not against resveratrol to augment the infarct volume. However, administration of nonselective NOS inhibitor was completely blocked the decrease infarction effect of resveratrol. The results suggested that eNOS and iNOS, but not nNOS, may be involve in the neuroprotective effect of resveratrol in rats subjected to FCI injury and NO plays a crucial role in the effects.

From this study, we presents the evidence that resveratrol increase NO production, attenuate the free radicals, and significantly protect rats brain tissue against FCI injury.

計畫成果自評：

This study had finished and prepared for publish.