

의학 박사학위 논문

**Cardioprotective Effect of Onion  
Extract against Ischemic Injury in vitro  
and in vivo**

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**Cardioprotective Effect of Onion Extract against  
Ischemic Injury in vitro and in vivo**

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-ABSTRACT-

## **Cardioprotective Effect of Onion Extract against Ischemic injury in vitro and in vivo**

Cardiomyocyte apoptosis is one of the pathological features of ischemic heart disease (IHD) and is associated with thrombosis or other acute alterations of coronary atherosclerotic plaques. The mechanisms of these ischemia/reperfusion injuries have been suggested to involve reactive oxygen species (ROS). *Allium* family, including garlic and onion, are known to have a potential to reduce the detrimental effect of cardiovascular risk factors, and their beneficial roles are suggested to be associated with antioxidant mechanism. Compared to garlic, very few have been reported about the myocardial beneficial effect of onion. Based on these considerations, the present study was designed to test whether onion extract have cardioprotective effect against ischemic injury in H9c2 cell culture system and rat myocardial infarct model in vivo, and if so, whether the beneficial effect of onion is attributed to its antioxidant property. This study also investigated which component

of onion extract is involved in the beneficial effect of onion extract. In H9c2 cells, onion extract markedly elicited cardioprotective effect against hypoxia-induced apoptotic death, demonstrated by its inhibitory effect on hypoxia-induced cytochrome *c* release, caspase-3 activation, and increase in TUNEL-positive cells. Onion extract also inhibited elevation of ROS generation induced by hypoxia. From inhibitory effects of onion extract and a well-known antioxidant trolox on hypoxia-induced decrease in mitochondrial membrane potential, it is suggested that antiapoptotic effect of onion extract is contributed to its antioxidant property, resulting in the inhibition of downstream mitochondrial death pathway. Cardioprotective effect of onion extract has been further demonstrated in rat myocardial infarct model in vivo, showing that administration of onion extract decreased infarct size and TUNEL-positive cells. Among components of onion extract, a major component quercetin showed a remarkable protective effect against ischemic injury in vitro and in vivo. The profile of the cardioprotective effect is same as in the case of onion extract.

**In conclusion, the results from this study suggest that onion extract has a**

significant cardioprotective effect contributed to antioxidant activity of its major component quercetin and that the signaling cascade of its antiapoptotic effect involves inhibition of mitochondrial death pathways, including loss of mitochondrial membrane potential, cytochrome c release and activation of caspase-3.

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Key words : Onion, *Allium*, apoptosis, ROS, Ischemia/reperfusion.

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## LIST OF ABBREVIATION

ACSOs: Alk(en)yl cysteine sulphoxides

DCF-DA: 2, 7-dichlorofluorescin diacetate

DMEM: Dulbecco's modified Eagle;s medium

DMSO: Dimethyl sulfoxide

ECG: electrocardiogram

IHD: Ischemic heart disease

LDH: Lactate dehydrogenase

MCSO: (+)-S-methyl-L-cysteine sulphoxide

MI: Myocardial ischemic

MMP: Mitochondrial membrane potential

MnSOD: Manganese superoxide dismutase

PeCOS: trans-(+)-S-(propen-1-yl)-L-cysteine sulphoxide

PrCSO: (+)-S-propyl-L-cysteine sulphoxide

PVDF: Polyvinylidene difluoride

ROS: Reactive oxygen species

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TTC: triphenyl tetrazolium chloride

TUNEL: In situ terminal deoxynucleotidyl transferase UTP nick and labeling

# I. INTRODUCTION

## A. Myocardial ischemic injury

Ischemic heart disease (IHD) is a worldwide health problem although the incidence varies between populations and areas of the world. Myocardial ischemic (MI) injury results from severe impairments of the coronary blood supply usually produced by thrombosis or other acute alterations of coronary atherosclerotic plaques (Dhalla et al., 2000; Buja and Entman., 1998). The characteristic pattern of myocardial ischemic injury involves fluid and electrolyte alterations, with loss of  $K^+$  and  $Mg^{2+}$  and accumulation of water,  $Na^+$ ,  $Cl^-$ ,  $H^+$  (acidosis), and  $Ca^{2+}$  cytoplasmic, organellar, and cellular swelling with plasma membrane blebbing; and margination and clumping of nuclear chromatin (Buja and Willerson., 1988). These cellular changes are due to progressive impairment of membrane composition, structure, and function (Buja et al., 1993). The transition from reversible to irreversible injury is characterized by the development of a severe membrane permeability defect that allows the unregulated influx of divalent and trivalent cations, including  $Ca^{2+}$ . Subsequently, the swollen cells develop physical defects in their cell membranes and rupture. These features of cell injury with cell swelling have been shown to involve cardiac myocytes subjected to hypoxia in vitro and cardiac muscle during the evolution of myocardial infarction in vivo (Reimer & Ideker., 1987).

MI injury involves necrosis and apoptosis. For a long time, necrosis was regarded

as the sole cause of cell death in MI injury. Recent studies indicate that apoptosis also plays an important role in the process of myocyte damage subsequent to myocardial infarction (Krijnen et al., 2002; Majno and Joris., 1995). It is now believed that necrosis is not the only form of cell death in cardiomyocytes. Cell death in cardiac can occur in a destructive, uncontrolled manner via necrosis or by a highly regulated programmed cell suicide mechanism termed apoptosis. Necrosis and apoptosis are two distinct forms of lethal cell injury (Hetts SW., 1998). Apoptosis is a highly regulated and energy-requiring process. Unlike necrosis, which is a passive and unregulated process, apoptosis is energy dependent and highly regulated. Also, in contrast to apoptosis, necrosis is a violent, irreversible, and non-regulated process of cell killing.

Cardiomyocyte apoptosis has recently been shown to occur in ischemic and reperfused myocardium both in humans (Scarabelli et al., 2001) and in animal models (Bialik et al., 1997). Many studies have demonstrated that apoptosis was implicated in experimental MI models (Fliss and Gattinger., 1996). The role of apoptosis in MI injury has recently been addressed in rat and rabbit animal models where reperfusion was shown to accelerate the occurrence of apoptotic cell death in cardiomyocytes (Haunstetter and Izumo., 1998).

## **B. Effect of onion (*Allium cepa*) on cardiovascular disease**

There has been an increasing recognition that certain natural substances have the potential to reduce the detrimental effect of a number of cardiovascular risk factors

(Galmarini et al., 2001; Craig., 1997; Riemersma et al., 2001). The use of natural substances has become more widespread over the past few years, natural substances may have fewer side effects than do pharmaceuticals and by their ready availability to the public without prescriptions. Members of *Allium* family, especially garlic and onion, have been used as a traditional medicine to treat a variety of diseases (Lanzotti., 2006; Chen., 2000; Block., 1985). *Alliums* such as garlic have been studied extensively for their health benefits (Milner., 1996; Warshafsky et al., 1993; Turner., 1994). Several *Allium* foods have been shown to reduce risks and modulate metabolism to favor the prevention of cardiovascular diseases (Banerjee et al., 2002; Maulik et al., 2002; Isensee et al., 1993)

Onion (*Allium cepa*) has long been used widely not only as flavoring agent but also as a folk medication (Kendler., 1987). Recent studies have validated many of the medicinal properties attributed to onion and its potential to lower the risk of cardiovascular disease (Galmarini., 2000; Morimitsu., 1990). Consumption of onion and related *Alliums* is associated with antioxidant (Geng Z & Lau B., 1997), antithrombotic (Bordia et al., 1998), antihyperlipidemic (Berthold et al., 1988), antihypertensive (Al-Quttan et al., 1999) and antiplatelet aggregation (Chen et al., 2000), contributing to decreased risk of cardiovascular disease. Extracts of Welsh onion (*Allium fistulosum*) can also modulate vascular responses in aorta (Chen et al., 1999). Raw extracts of this species lower systolic blood pressure and prolong bleeding times in rats possibly through inhibition of platelet function and suppression of thromboxane production (Chen et al., 2000). Similarly, intake of dietary

flavonoids and flavones has been reported to be inversely associated with risk of cardiovascular disease in several epidemiological studies (Yochum et al., 1999). Cardiovascular effect of onion is suggested to be attributed to a property of the organosulphur compound. Clearly there are many claims on cardiovascular protection of *Alliums*, however, most, with the exception of garlic, have not received any rigorous scientific investigation.

### **C. Chemical composition of onion and the key health compounds**

Among members of *Allium* family, onion (*Allium cepa*), leek (*Allium ampeloprasum*) and garlic (*Allium sativum*) contains active components that benefit metabolic and cardiovascular disease (Jones et al., 2004). Onions are rich in two chemical groups that have perceived health benefits to man. There are the flavonoids and the alk(en)yl cysteine sulphoxides (ACSOs). Two flavonoid subgroups are found in onion, the anthocyanins, which impart a red/purple color to some varieties and flavanols such as quercetin and its derivatives responsible for the yellow flesh and brown skins of many other varieties (Leighton et al., 1997). The ACSOs are the flavour precursors, which when cleaved by the enzyme alliinase, generate the characteristic odour and taste of onion (Griffiths et al., 2002). The flavour of onion is mainly due to sulphur-containing compounds, formed by the cleavage of three S-alk(en)yl-L-cysteine sulphoxides (ACSOs) by alliin alkyl-sulphenate-lyase (alliinase). There ACSOs are (+)-S-methyl-L-cysteine sulphoxide (MCSO, methiin), (+)-S-propyl-L-cysteine sulphoxide (PrCSO, propiin) and trans-(+)-S-(propen-1-yl)-L-



cysteine sulphoxide (1-PeCSO, isoalliin) (Kopsell et al., 1999; Kubec et al., 2002).

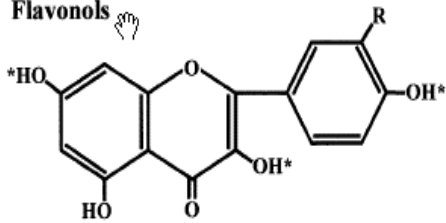
The major flavour compounds are generated by the spontaneous reactions undergone by S-alk(en)yl sulphenic acids among themselves and other compounds (Kubec & Musah., 2001). The results is a mixture of over 50 sulphur containing compounds including thiosulphinates, thiosulphonates, mono-, di- and tri-sulphides as well as specific compounds such as the lachrymatory or tear factor, thiopropanal S-oxide (Kubec et al., 2000).

Alliin alkyl-sulphenate-lyase (alliinase) catalyses the cleavage of ACSOs to give a sulphenic acid and  $\alpha$ -iminopropionic acid (Lancaster et al., 2000). Onion alliinase is a glycoprotein with a carbohydrate content of approximately 4.6% and comprising about 6% of total soluble protein of bulb tissue (Nock and Mazelis., 1987). In onion bulb, alliinase is located in the vacuole (Lancaster and Collin., 1981) in both monomeric and multimeric forms.

There are several suggestions for the role of alliinase in the plant. The most obvious is a defensive function since the ACSO breakdown products deter and are often lethal to, a wide range of pests. Alliinase may also have a role in remobilizing sulphur from ACSOs during condition of sulphur deprivation (Bacon et al., 1999), since the specific activity of the enzyme increases at lower sulphur levels in the soil (Lancaster et al., 2000). Flavonoids and ACSOs of onion may provide further benefit to the cardiovascular system through their abilities to inhibit ROS and lower serum lipid and cholesterol levels (Yin and Cheng., 1998). Additional constituents of intact onion include the following: steroidal glycosides, fructan, pectin, essential oil,

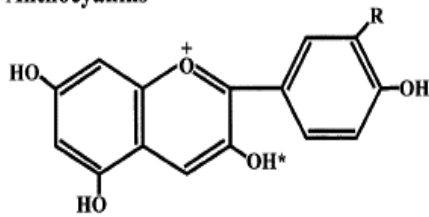
adenosine, vitamin C and E, biotin, nicotinic acid, phenolics and essential amino acids (Fenwick and Hanley., 1985; Kopsell et al., 2003 ). The importance of the constituents in explaining the health benefit of onion remains to be resolved.

### Flavonols



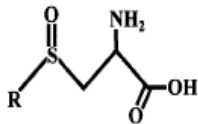
R = H    Kaempferol  
R = OH    Quercetin  
R = OCH<sub>3</sub>    Isorhamnetin  
\* = Sites of glycosylation

### Anthocyanins



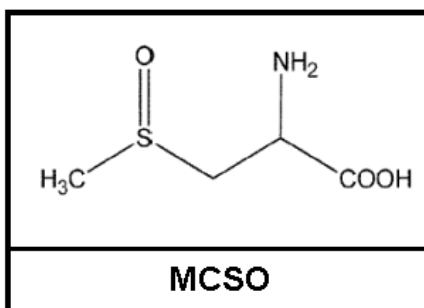
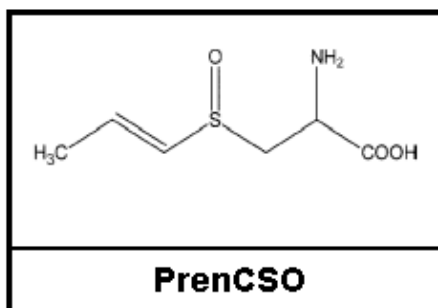
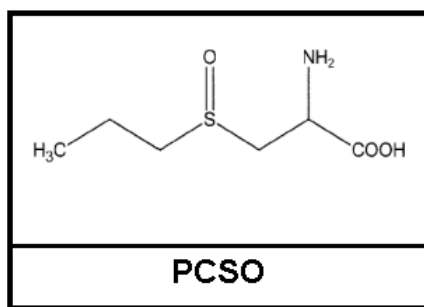
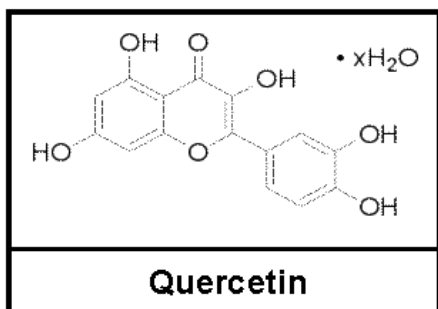
R = OH    Cyanidin  
R = OCH<sub>3</sub>    Peonidin  
\* = Site of glycosylation

### Alkyl cysteine sulphoxides



R = CH<sub>3</sub>    (+)-S-Methyl-L-cysteine sulphoxide  
R = CH<sub>3</sub>-CH<sub>2</sub>-CH<sub>2</sub>    (+)-S-Propyl-L-cysteine sulphoxide  
R = CH<sub>3</sub>-CH=CH    *Trans*-(+)-S-(Propen-1-yl)-L-cysteine sulphoxide

**Fig. 1. Beneficial health compounds in onion**



**Fig. 2. Structure of key health in onion**

## **II. Aims of Study**

In this study, it was investigated whether onion extract protect on ischemia-induced apoptotic cell death involving in ROS generation in H9c2 cell and myocardial infarction model.

### **A. Effect of onion extract on ischemia-induced cell death**

- 1) Does onion extract protect H9c2 cells against hypoxia-induced cell death?
- 2) Does onion extract protect H9c2 cells against hypoxia-induced ROS generation?
- 3) Does onion extract protect H9c2 cells against decreased MMP by hypoxia?
- 4) Does onion extract reduce against infarct size in rat myocardial infarct model?
- 5) Does onion extract reduce against apoptotic cell death in rat myocardial infarct model?

### **B. Effect of onion component on ischemia-induced cell death**

- 1) Do onion components protect H9c2 cells against hypoxia-induced cell death?
- 2) Do onion components protect H9c2 cells against hypoxia-induced ROS generation?
- 3) Do onion components protect H9c2 cells against decreased MMP by hypoxia?

- 4) Do onion components reduce against infarct size in rat myocardial infarct model?
- 5) Do onion components reduce against apoptotic cell death in rat myocardial infarct model?

### **III. METHODS**

#### **A. Cell culture and hypoxia system**

Heart derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured before experimentation in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 Mm glucose supplementation with 10% fetal bovine serum. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37°C with a humidified atmosphere of 5% CO<sub>2</sub>, 10% H<sub>2</sub>, and 85% N<sub>2</sub>. In the anaerobic chamber, the culture medium was replaced with serum-free, glucose-free DMEM that had been saturated with N<sub>2</sub> gas for 1 h. Normoxic incubation of the cells in the serum-free DMEM was conducted in a water-jacked incubator gassed with 95% air and 5% CO<sub>2</sub> at 37°C.

#### **B. Onion extract treatment**

In vitro, onion extract was dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO was 0.1%, and this concentration of DMSO was found to have no effect on H9c2 cell viability. H9c2 cells were treated with inhibitors 30 min before and during hypoxia.

In vivo, Chronic oral administration in rats were administered orally 1 ml vehicle (0.9% NaCl) or various doses (0.1, 1, 3 or 10 g/kg) of onion extract , daily from the

day of operation till 14 days after. I.V. injection was dissolved in saline (Sigma, USA) and administered intravenously by a single bolus injection (1, 3 or 10 g/kg) 30 min before the LAD occlusion.

### **C. Lactate dehydrogenase (LDH) assay**

Cell death was assessed by measuring the lactate dehydrogenase (LDH) released into the culture medium. This enzyme located in the cytoplasm in viable cells, is released to the media by cells that have lost membrane integrity. Assessment of LDH activity in culture media was performed by measuring the decrease in absorbance at 340 nm due to the conversion of enzyme cofactor NADH to NAD<sup>+</sup>. Briefly, 25 $\mu$ l aliquots of culture media were collected and dissolved in NADH (0.25 mg in phosphate buffer) solution. Pyruvate buffer (22.7 mM sodium pyruvate in phosphate buffer saline, 0.1 M, Ph = 7.4) was then added to the samples. Maximal LDH release was measured by 0.1% triton X-100. Values were expressed relative to the measurements from maximal neuronal LDH.

### **D. In situ terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL)**

To examine the extent of apoptotic cell death, we performed TUNEL-staining after 8 h of hypoxia. In situ labeling of fragmented DNA was performed by TUNEL assay with the commercially available Apop Tag Plus kit (Oncor, Gaithersburg, MD, USA). In vitro, monolayer of H9c2 cells were grown on 24-well plates and fixed with 4%



paraformaldehyde. Then nucleosome-sized DNA fragments were tailed with digoxigenin nucleotide and reacted with fluorescein-conjugated antidigoxigenin antibodies. In vivo, 10µm tissue sections were cut in a cryotome cryostat (Life sciences International, England) from embedded heart tissue. Sections of control, sham operated, and 45 min ischemia-12 h reperfusion induction animals were subjected to the TUNEL assay using an in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The nucleus was counterstained with hematoxyline. The percent cell death was calculated from the number of TUNEL-positive cells divided by total cell count.

#### **E. Caspase -3 activity assay**

The cells were lysed with lysis buffer (10 mM Tris/HCl, 0.32 M Sucrose, 1 mM PMSF, 1% Triton X-100, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM EDTA, and 10 mM DTT, pH 8.0) for 30 min and the lysates were centrifuged (10,000 × g for 5 min at 4°C). Samples (200 µg) of the extracted protein were incubated with the reaction buffer (100 mM HEPES, 10% sucrose, 0.1% 3-[3-cholamidopropylammonio]-1-propanesulfonate (CHAPS), pH 7.5, 10 mM DTT and 10 µg/ml leupeptin) to 100 µl volume containing 200 µM Ac-DEVD-*p*-Na (Biomol, Plymouth meeting, PA, USA). Enzyme-catalyzed release of *p*-nitroanilide was measured at 405 nm using microplate reader (Molecular Devices, Palo Aldo, CA, USA).

## **F. Separation of cytosolic and mitochondrial fractions**

The cells for lysate preparation were washed twice with ice-cold PBS and collected by centrifugation at  $1,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The cell pellets were washed once with ice-cold PBS and resuspended in lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1.0 mM sodium EDTA, 1.0 mM sodium EGTA, 1.0 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose) supplemented with protease inhibitors (10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  aprotinin). The cells were then homogenized in a glass homogenizer, and the nuclei and cell debris were removed by centrifugation at  $1,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatants were further centrifuged at  $10,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , and the resulting mitochondrial pellets were resuspended in lysis buffer. The supernatants created from the  $10,000 \times g$  centrifugation were centrifuged once more at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ , and the supernatant was collected and designated the cytosolic fraction.

## **G. Western blot analysis for cytochrome *c* release**

To quantify cytochrome *c* release, Western blot analysis in the mitochondrial and the cytosolic fractions was performed. A quantity of 40  $\mu\text{g}$  protein of either the cytosolic fraction or the mitochondrial fraction was separated on 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MD). The membrane was blocked with 5% nonfat dry milk for 90 min, and incubated overnight

with a primary mouse anti-cytochrome c monoclonal antibody (BD Biosciences, Lexington, KY, USA). The membrane was then incubated with a secondary anti-IgG antibody conjugated with alkaline phosphatase for 4 h, and the cytochrome c band was visualized using the NBT/BCIP method (Sigma-Aldrich, St. Louis, MO, USA).

We verified the mitochondrial fraction with the mitochondrial marker cytochrome oxidase subunit IV (COX IV, Molecular Probes, Eugene, OR, USA), and cytosolic fractions with the cytosol marker actin (Sigma, St. Louis, MO, USA).

#### **H. Intracellular ROS measurement**

5(6)-Chloroemthyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H<sub>2</sub>DCFDA; Molecular Probe) is an ROS-sensitive probe that can be used to detect oxidative activity in living cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihydrofluorescein derivative. Its thiol-reactive chloromethyl group reacts with intracellular GSH and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell. CM-H<sub>2</sub>DCFDA is prepared in HCSS immediately before loading. H9c2 cells cultured on microwell glass-bottomed culture dishes were loaded with the dye (1µg/ml) immediately after hypoxia. The cultures were then incubated in the dark at 37°C for 30 min. After they were washed, the cells were allowed to recover at 37°C for 20 min. The plates were examined immediately under a laser scanning confocal microscope (Olympus 5100, Japan).

## **I. Mitochondrial membrane potential ( $\Delta\Psi_m$ ) measurement**

$\Delta\Psi_m$  was assayed by measuring the accumulation of rhodamine 123 (Molecular probes, Eugene, OR, USA), a membrane-permeable cationic fluorescent dye (Emaus et al., 1986).

H9c2 cells were incubated with 1  $\mu\text{M}$  rhodamine 123 at 37° for 30 min. After incubation at 37°, cells were washed three times with DMSO and were observed at 530 nm under confocal microscope (Olympus 5100, Japan).

## **J. Rat myocardial infarct model**

Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). The trachea was intubated and the rats were mechanically ventilated with room air using a small rodent ventilator (Model 683, Harvard Apparatus) at a rate of 60 cycles per minute and a tidal volume of 1 ml/100g body weight. The left femoral artery was cannulated for measurement of mean arterial pressure and heart rate. The left femoral vein was catheterized for drug administration, and the electrocardiogram (ECG) was recorded with the standard Lead II limb leads. Body temperature was maintained at 37°C using a water circulating heating blanket.

A left thoracotomy was performed, and the heart was exposed through the fifth intercostal space. The pericardium was incised and the heart was gently exteriorized by pressure on the chest, and a 4-0 silk suture was placed around the left main coronary artery near its origin beneath the left atrial appendage. The heart was repositioned in the thoracic cavity with the ligature ends exteriorized and

passed through a small length of PE100 tubing to form a snare. For coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery and a bulldog hemostat was applied to the snare for 25 min, resulting in coronary artery occlusion. Myocardial ischemia was confirmed by the presence of ECG changes and by the visual assessment of regional cyanosis and dyskinesia of ischemic region of the left ventricle. Reperfusion was verified by the presence of reperfusion arrhythmias.

#### **K. Measurement of myocardial infarct size**

Immediately before sacrificing the rat, the branch of the left anterior descending coronary artery was ligated again and Evans blue was quickly injected into the left ventricular chamber to distinguish darkened normal myocardium from unstained area at risk. After killing the animal, the right ventricle was dissected and the left ventricle was transversely sectioned in five slices, each portion was weighed, then washed and incubated for 10 min, in a solution of triphenyl tetrazolium chloride (TTC) (Sigma). Salvaged myocardium within the area at risk stained brick red, whereas coloration of the infarct mass remained unaltered. Slices were then photographed (Gel doc 2000, BIORAD) and later analyzed via computerized planimetry (BAS 1500, Fuji film) to evaluate the composition of each heart with respect to normal, area at risk and infarcted left ventricular zones. From these procedures a mathematical representation of total infarct and risk zone sizes were calculated in control and treated groups.

## **L. Statistical analysis**

All data were expressed as mean $\pm$ S.D. The numerical data were compared by a Student's *t*-test for unpaired observations between the two groups. A *P*-value of  $<0.05$  was considered significant.

## IV. RESULTS

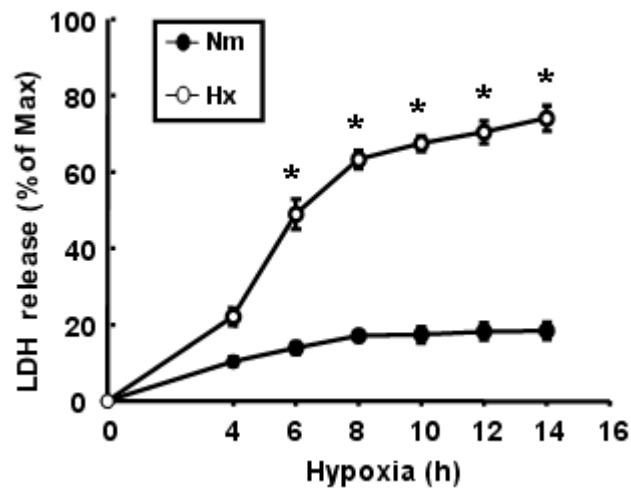
### A. Effect of onion extract against hypoxia-induced cell death

To investigate the effect of onion extract on hypoxia-induced cell death, we measured LDH activity released during hypoxia in the presence or absence of onion extract. In H9c2 cells exposed to hypoxia for 8 h, LDH release was increased up to about  $62.8 \pm 6.18\%$  compared to that in the normoxia ( $11.8 \pm 1.64\%$ ). As shown in Fig. 3, hypoxia-induced cell death ( $62.8 \pm 6.18\%$ ) was inhibited by treatment with 0.01, 0.05 and 0.1 g/ml onion extract ( $31.1 \pm 1.31\%$ ,  $13.9 \pm 0.67$  and  $14.8 \pm 2.25\%$ , respectively).

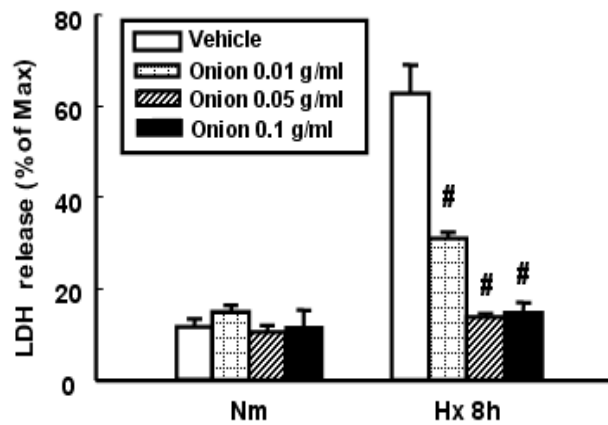
### B. Effect of onion extract against hypoxia-induced ROS generation

To investigate the effect of onion extract on hypoxia-induced ROS generation, we assessed the changes of dichlorofluorescein (DCF-DA) fluorescence using confocal microscope. The ROS generation during hypoxia began to increase at 15 min after hypoxia and further increased to the maximum level (about 5.7 fold) at 60 min after hypoxia (Fig. 4). The ROS generation during hypoxia 60 min was dose-dependently decreased by treatment with onion extract of 0.05 and 0.1 g/ml (about 3.5 fold and 3.3 fold, respectively).

(A)



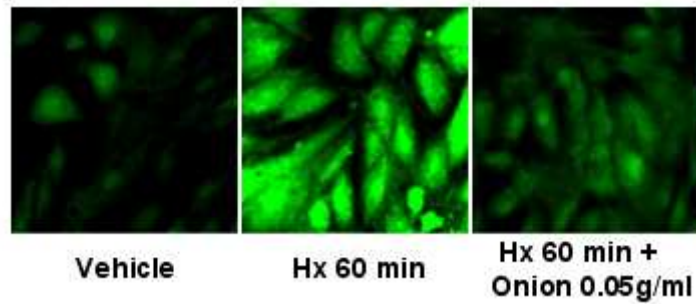
(B)



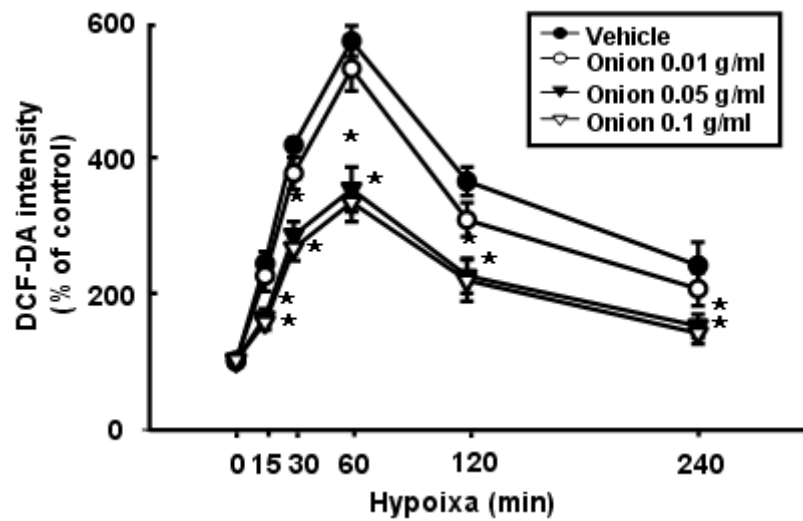
**Fig 3. Effect of onion extract on LDH release during hypoxia in H9c2 cells.** (A) Time course of LDH release during hypoxia (Hx). \* P<0.05 vs. Normoxia (Nm). (B) Effect of onion extract on Hx-induced LDH release. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during hypoxia (Hx). All values are mean  $\pm$  SD (n= 4). # P <0.05 vs. Hx-Vehicle.



(A)



(B)



**Fig. 4. Effect of onion extract on hypoxia-induced reactive oxygen species (ROS) generation in H9c2 cells.** (A) Representative images obtained by DCF-DA confocal laser microscopy. (B) Effect of onion extract on Hx-induced ROS generation determined by confocal images of dichlorofluorescein (DCF-DA) staining. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during hypoxia (Hx). All data represent mean  $\pm$  SD (n = 4). \* P<0.05 vs. Hx-Vehicle.

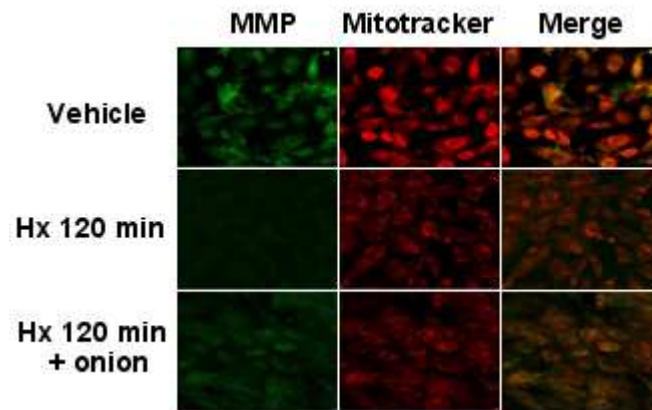
### **C. Effect of onion extract on hypoxia-induced reduction in mitochondrial membrane potential ( $\Delta\Psi_m$ )**

To examine whether preservation of mitochondrial membrane potential is associated with cardioprotective effect of onion extract, we assessed the changes of rhodamine 123 fluorescence in the H9c2 cells exposed to hypoxia using confocal microscope. Fig. 5. shows the time-dependent changes in rhodamine 123 fluorescence. The  $\Delta\Psi_m$  was remarkably reduced by  $51.4\pm 1.45\%$  as early as after 2 h of hypoxia and reached a maximum level ( $39.5\pm 2.42\%$ ) after 4 h of hypoxia compared to normoxic control. The  $\Delta\Psi_m$  reduction after 4 h of hypoxia ( $39.5\pm 2.42\%$ ) was completely abolished by 0.05 and 0.1 g/ml onion extract ( $62.5\pm 4.46\%$  and  $61.7\pm 4.96\%$ , respectively)

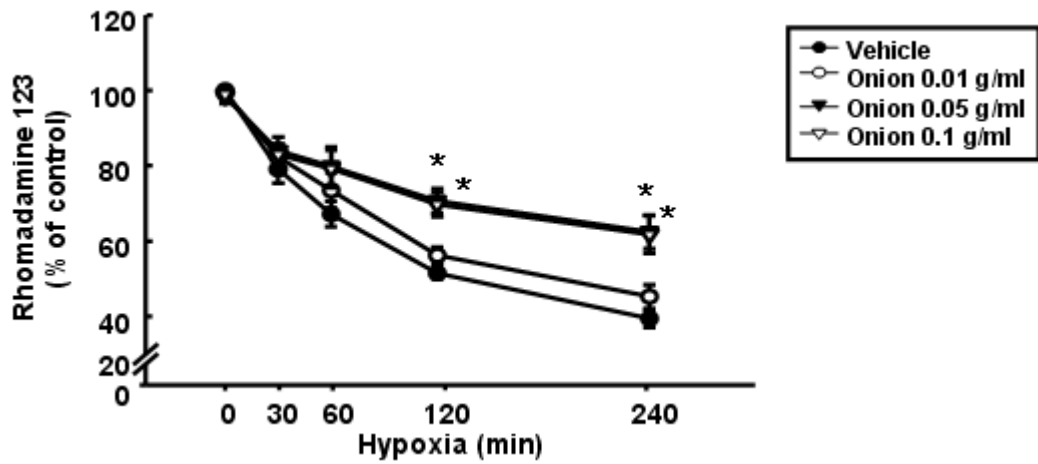
### **D. Effect of onion extract on cytosolic release of cytochrome *c* by hypoxia**

To investigate the effect of onion extract on the release of cytochrome *c* from mitochondria to cytosol, the cytosolic release of cytochrome *c* was examined by western blotting in both cytosolic and mitochondrial fractions. The cytosolic release of cytochrome *c* was dramatically increased after 8 h of hypoxia. However, it was markedly inhibited by treatment with 0.05 g/ml onion extract (Fig. 7).

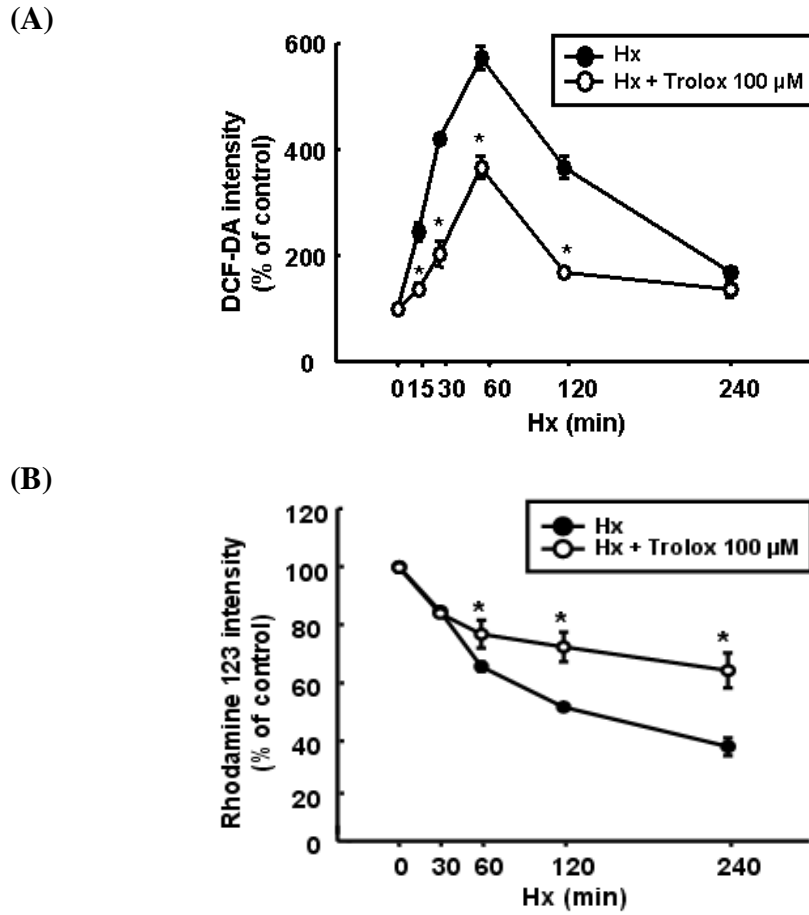
(A)



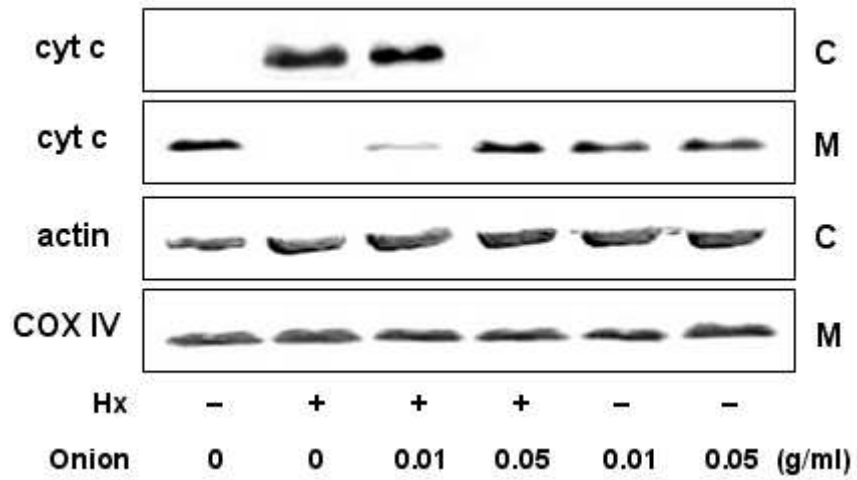
(B)



**Fig. 5. Effect of onion extract on hypoxia-induced mitochondrial membrane potential ( $\Delta\Psi_m$ ) reduction in H9c2 cells. (A)** Representative images of rhodamine 123 intensity obtained by confocal laser microscopy. **(B)** Effect of onion extract on Hx-induced  $\Delta\Psi_m$  reduction. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during hypoxia. All data represent mean  $\pm$  SD (n = 4). \* P<0.05 vs. Hx-Vehicle.



**Fig. 6. Effect of trolox on hypoxia-induced reactive oxygen species (ROS) generation and mitochondrial membrane potential ( $\Delta\Psi_m$ ) reduction. (A) Effect of trolox on Hx-induced ROS generation determined by confocal images of dichlorofluorescein (DCF-DA) staining. H9c2 cells were treated with trolox (100  $\mu$ M) 30 min before and during hypoxia. (B) Effect of trolox on Hx-induced  $\Delta\Psi_m$  reduction determined by confocal laser microscopy image of rhodamine 123 intensity. H9c2 cells were treated with trolox (100  $\mu$ M) 30 min before and during hypoxia. All values are mean  $\pm$  SD (n= 4). \*P <0.05 vs. Hx-Vehicl**



**Fig. 7. Western blot analysis for cytochrome *c* during hypoxia in H9c2 cells.** Western blots for cytochrome *c* were detected in the cytosolic (C) and mitochondrial (M) fractions 6 h after hypoxic insult. H9c2 cells were treated with onion extract (0.01 and 0.05 g/ml, respectively) 30 min before and during hypoxia.

### **E. Activation of caspase-3 during hypoxia**

To investigate the effect of onion extract on hypoxia-induced cell death, we measured caspase-3 activity. As shown in Fig. 8A, hypoxia increased caspase-3 activity ( $213.8 \pm 2.80\%$ ) compared to the normoxic control (100%). The treatment of cells with 0.05 and 0.1 g/ml onion extract ( $142.5 \pm 4.10\%$  and  $116.2 \pm 3.70\%$ , respectively) markedly decreased the caspase-3 activity nearly up to the level of normoxia vehicle.

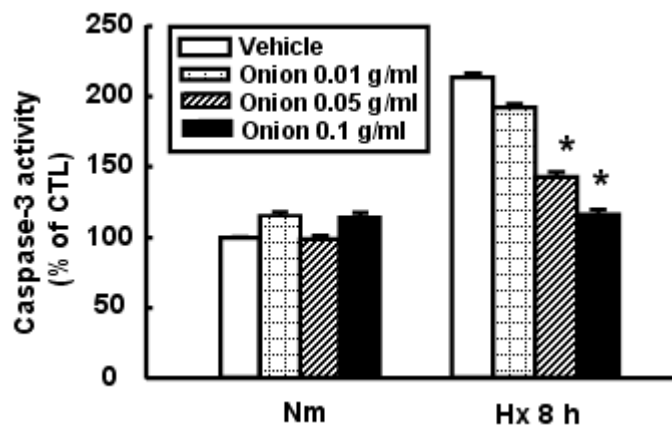
### **F. Effect of onion extract against hypoxia-induced apoptosis**

To investigate the effect of onion extract on hypoxia-induced apoptotic cell death, we performed TUNEL staining. The number of TUNEL-positive cells was  $47.0 \pm 0.40\%$  in vehicle-treated cells exposed to hypoxia for 8 h. When the cells were treated with 0.05 and 0.1 g/ml of onion extract, the number of TUNEL-positive cells after 8 h of hypoxia was significantly decreased ( $18.3\% \pm 3.50\%$  and  $7.00\% \pm 4.10\%$ , respectively).

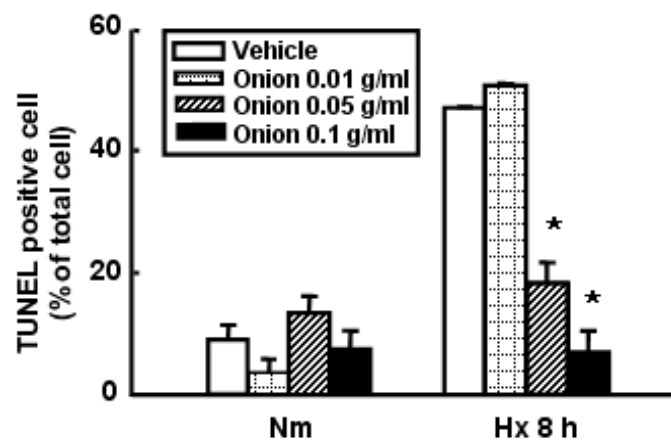
### **G. Effect of onion extract against BSO-induced cell death**

To investigate the effect of onion extract on BSO-induced cell death, we measured LDH activity released during hypoxia in the presence or absence of onion extract. In H9c2 cells exposed to BSO for 24 h, LDH release was increased up to about  $57.2 \pm 7.00\%$  compared to that in the normoxic condition ( $20.9 \pm 1.29\%$ ). As shown in Fig. 9, BSO-induced cell death ( $57.2 \pm 7.00\%$ ) was inhibited by treatment with 0.05 and 0.1 g/ml onion extract ( $30.9 \pm 1.23\%$  and  $28.5 \pm 2.50\%$ , respectively).

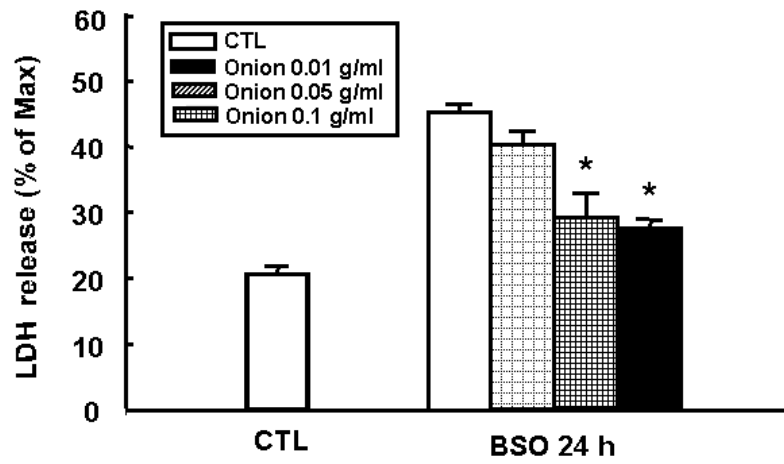
(A)



(B)



**Fig 8. Effect of onion extract on apoptotic cell death during hypoxia in H9c2 cells.** (A) Effect of onion extract on hypoxia-induced caspase-3 activation. (B) Effect of onion extract on TUNEL-positive cells increased by hypoxia (Hx). TUNEL-positive cells was evaluated as a percentage of total cell counts. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during hypoxia. All data represent mean  $\pm$  SD (n = 4). \* P<0.05 vs. Hx-Vehicle. Nm means normoxia.



**Fig. 9. Effect of onion extract on LDH release during BSO injury.** H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during BSO injury. All values are mean  $\pm$  SD (n=4). \* P<0.05 vs. BSO-Vehicle. CTL means control.



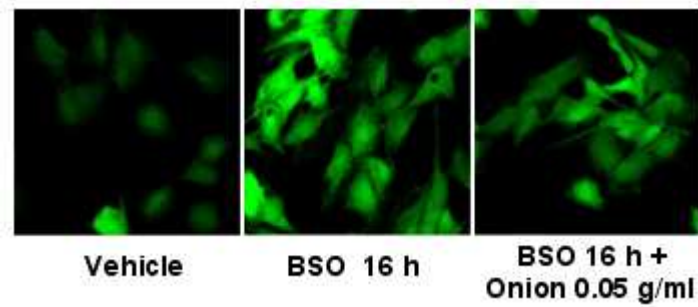
## **H. Effect of onion extract against BSO-induced ROS generation**

To investigate the effect of onion extract on BSO-induced ROS generation, we assessed the change of dichlorofluorescein (DCF-DA) fluorescence in the cells exposed to BSO using confocal microscope. Fig. 10. shows the time-dependent changes in DCF-DA fluorescence. The ROS generation was remarkably increased above 3.8 fold by as early as after 16 h of BSO and dose-dependently decreased by treatment with 0.05 and 0.1 g/ml onion extract.

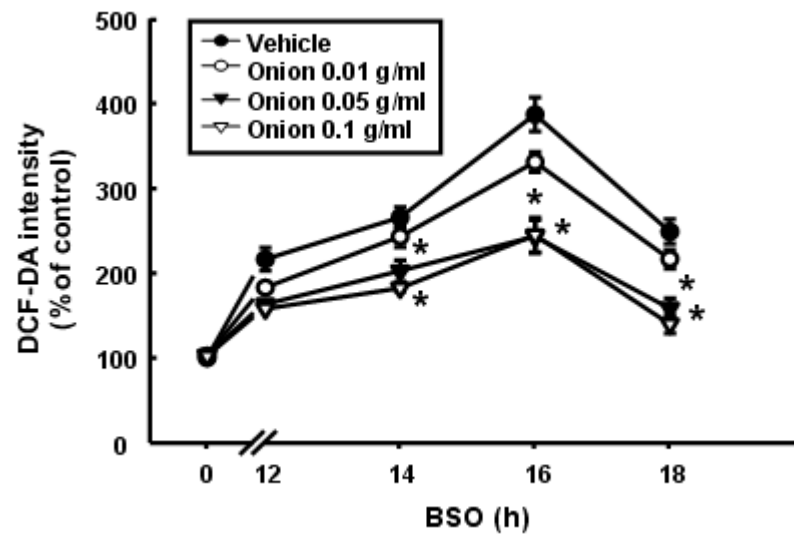
## **I. Effect of onion extract on BSO-induced reduction in mitochondrial membrane potential ( $\Delta\Psi_m$ )**

To examine whether preservation of mitochondrial membrane potential ( $\Delta\Psi_m$ ) is associated with cardioprotective effect of onion extract, we assessed the change of rhodamine 123 fluorescence in the cells exposed to hypoxia using confocal microscope. Fig. 11 shows the time-dependent changes in rhodamine 123 fluorescence. The  $\Delta\Psi_m$  was remarkably reduced by  $55.6\pm 3.54\%$  as early as after 16 h of BSO and reached a maximum level ( $38.3\pm 3.62\%$ ) after 18 h of BSO treatment compared to that on normoxic control. The  $\Delta\Psi_m$  reduction after 4 h of BSO was completely abolished by the treatment with 0.05 and 0.1 g/ml onion extract ( $56.3\pm 3.83\%$  and  $55.7\pm 5.72\%$ , respectively).

(A)

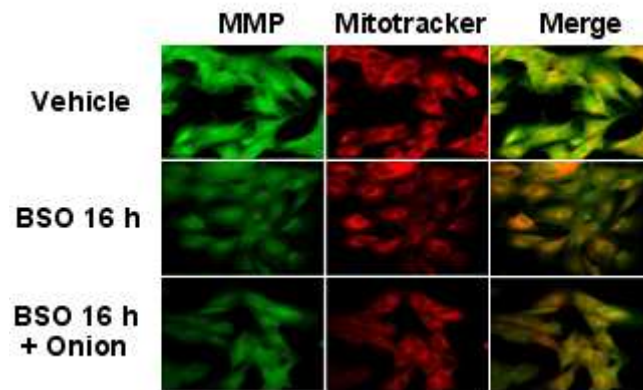


(B)

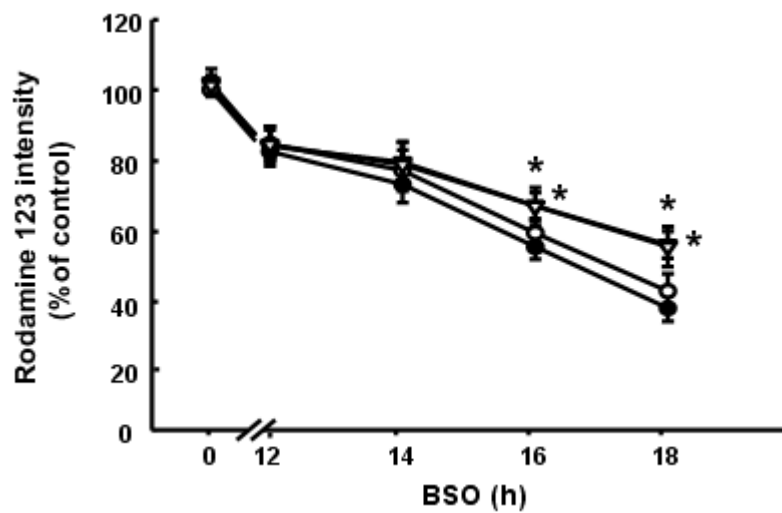


**Fig. 10. Effect of onion extract on BSO-induced reactive oxygen species (ROS) generation in H9c2 cells.** (A) Representative images obtained by DCF-DA confocal laser microscopy. (B) Effect of onion extract on BSO-induced ROS generation determined by confocal images of dichlorofluorescein (DCF-DA) staining. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during BSO. All values are mean  $\pm$  SD (n= 4). \*P <0.05 vs. BSO-Vehicle.

(A)



(B)



**Fig. 11. Effect of onion extract on BSO-induced mitochondrial membrane potential ( $\Delta\Psi_m$ ) reduction in H9c2 cells. (A)** Representative images of rhodamine 123 intensity obtained by confocal laser microscopy. **(B)** Effect of onion extract on BSO-induced  $\Delta\Psi_m$  reduction. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during BSO. All values are mean  $\pm$  SD (n= 4). \*P <0.05 vs. BSO-Vehicle.

## **J. Effect of onion extract against BSO-induced apoptosis**

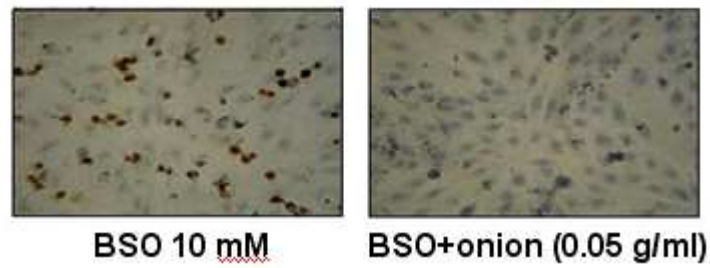
To investigate the effect of onion extract on BSO-induced apoptotic cell death, we performed TUNEL-staining. The number of TUNEL-positive cells was  $37.1 \pm 2.50\%$  in vehicle-treated cells exposed to BSO for 18 h. When the cells were treated with 0.05 and 0.1 g/ml of onion extract, the number of TUNEL-positive cells was significantly decreased after BSO treatment for 18 h ( $4.30 \pm 2.20\%$  and  $2.10 \pm 1.40\%$ , respectively).

## **K. Effect of onion extract on infarct size in rat myocardial infarction model**

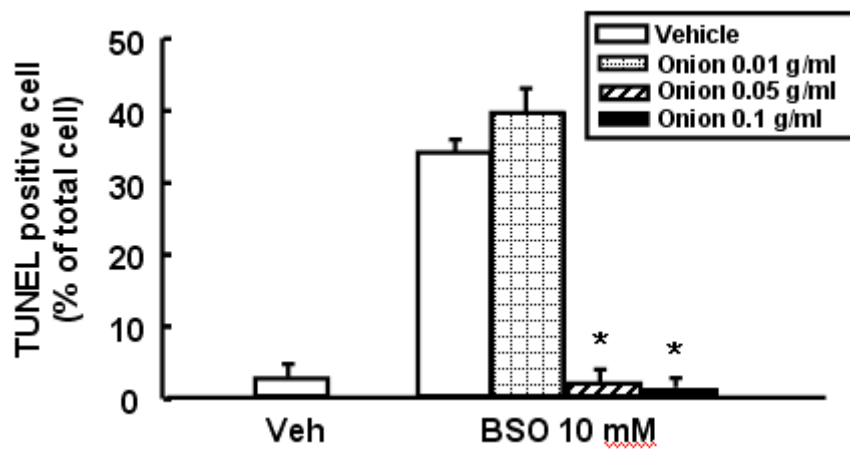
To investigate the effect of onion extract on ischemia/reperfusion induced infarct size, we performed TTC-staining. In the i.v. vehicle-treated group, ischemia (45 min) followed by reperfusion (90 min) resulted in an infarct size of  $64.6 \pm 2.28\%$  of the area at risk. Onion extract reduced the myocardial infarct size in a dose dependent manner ( $53.6 \pm 1.64\%$ ,  $45.8 \pm 1.79\%$  and  $31.8 \pm 1.31\%$  at 0.1, 1.0 and 10.0 g/k, respectively).

In the p.o. vehicle-treated group, In the i.v. vehicle-treated group, ischemia (45 min) followed by reperfusion (90 min) resulted in an infarct size of  $63.3 \pm 1.84\%$  of the area at risk. Chronic oral intake of onion extract (1.0, 3.0 and 10.0 g/kg) significantly reduced ( $55.0 \pm 1.77\%$ ,  $52.8 \pm 2.09\%$  and  $47.0 \pm 2.00$ , repectively) myocardial infarct size (Fig. 14).

(A)

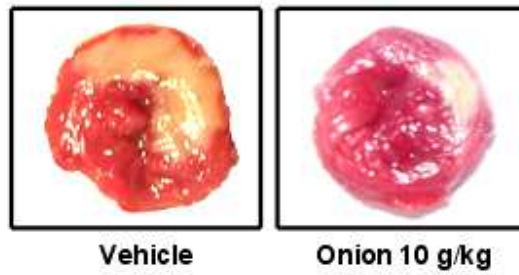


(B)

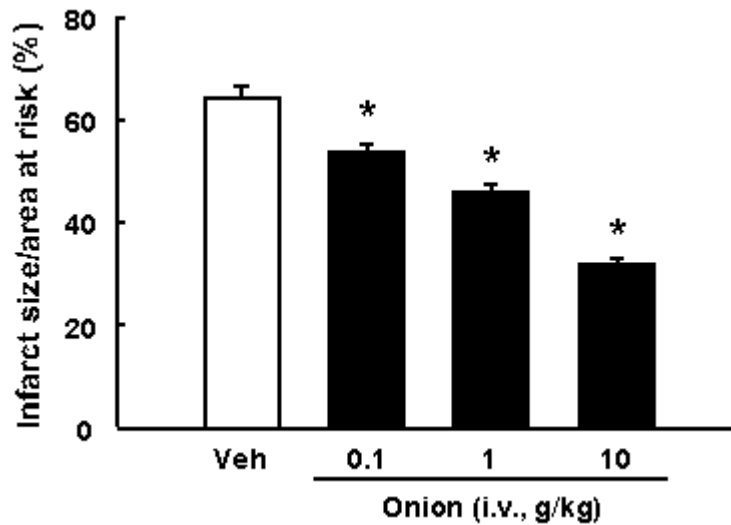


**Fig. 12. Effect of onion extract on TUNEL-positive cells during BSO injury.** (A) Representative photomicrographs of TUNEL-positive cells for DNA breaks. (B) Quantitative analysis of TUNEL-positive cells evaluated as a percentage of total cell counts. H9c2 cells were treated with onion extract (0.01, 0.05 and 0.1 g/ml) 30 min before and during hypoxia. All data represent mean  $\pm$  SD (n = 4). \* P<0.05 vs. Hx-Vehicle (Veh).

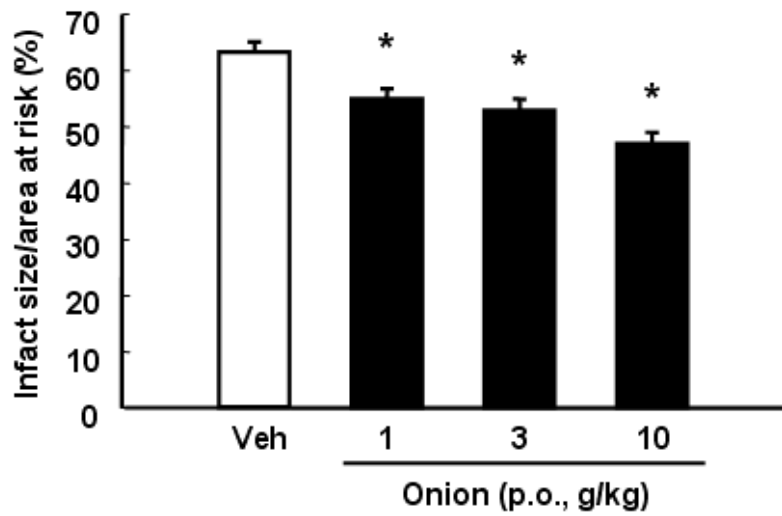
(A)



(B)



**Fig. 13. Effect of onion extract (i.v.) on infarct size in rat myocardial infarct model** (A) Representative photomicrographs of left ventricular section. (B) Quantitative assessment of infarct size/AAR(area at risk). Myocardial infarct was induced by 45 min ischemia/90 min reperfusion. Onion extracts (0.1, 1.0, 3.0 and 10 g/kg) were intravenously administered 20 min before occlusion. All values are mean  $\pm$  SEM (n= 8). \*p<0.05 vs. Vehicle (Veh).



**Fig. 14. Effect of onion extract (p.o.) on infarct size in rat myocardial infarct model.** Myocardial infarct was induced by 45 min ischemia/90 min reperfusion. Onion extracts (1.0, 3.0 and 10.0 g/kg) were administered orally once daily for 2 weeks. All values are mean  $\pm$  SEM (n= 8). \*p<0.05 vs. Vehicle (Veh).

#### **L. Effect of onion extract against ischemia-reperfusion induced apoptosis**

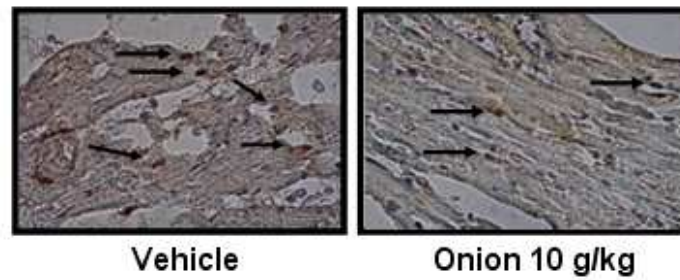
To investigate the effect of onion extract on ischemia/reperfusion induced apoptotic cell death, we performed TUNEL-staining. Occlusion of the left main coronary artery for a period of 45 minutes followed by 12 h of reperfusion resulted in substantial injury to the myocardium. As shown in Fig. 15, the amounts of TUNEL-positive myocytes in onion extract-treated (1,0 and 10 g/kg) hearts were significantly less than that in control ischemia-reperfused hearts ( $8.29\pm 0.73\%$  and  $4.61\pm 0.57$  vs.  $14.34\pm 1.93\%$ ).

#### **M. Effect of onion components against hypoxia-induced cell death**

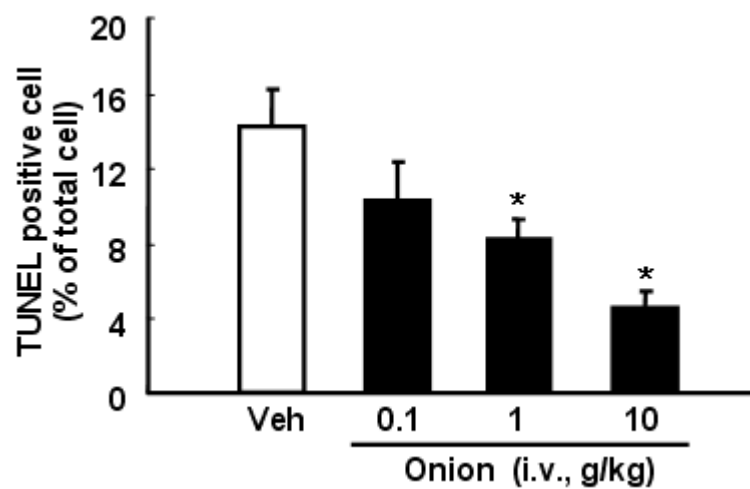
To investigate the effects of onion components on hypoxia-induced cell death, we measured LDH activities released during hypoxia after treatment with quercetin, PCSO, MCSO and prenCSO (Fig. 16). LDH release were  $58.3\pm 1.35\%$  in vehicle-treated cells exposed to hypoxia for 8 h. Treatment with low dose of PCSO, MCSO and prenCSO did not reduce hypoxia induced LDH release. On the other hand, when were treated with low dose (0.1 and 1.0  $\mu\text{g/ml}$ ) of quercetin, LDH release after 8 h of hypoxia was significantly decreased ( $47.52\%\pm 4.3\%$  and  $42.2\%\pm 3.67\%$ , respectively).



**A**



**B**

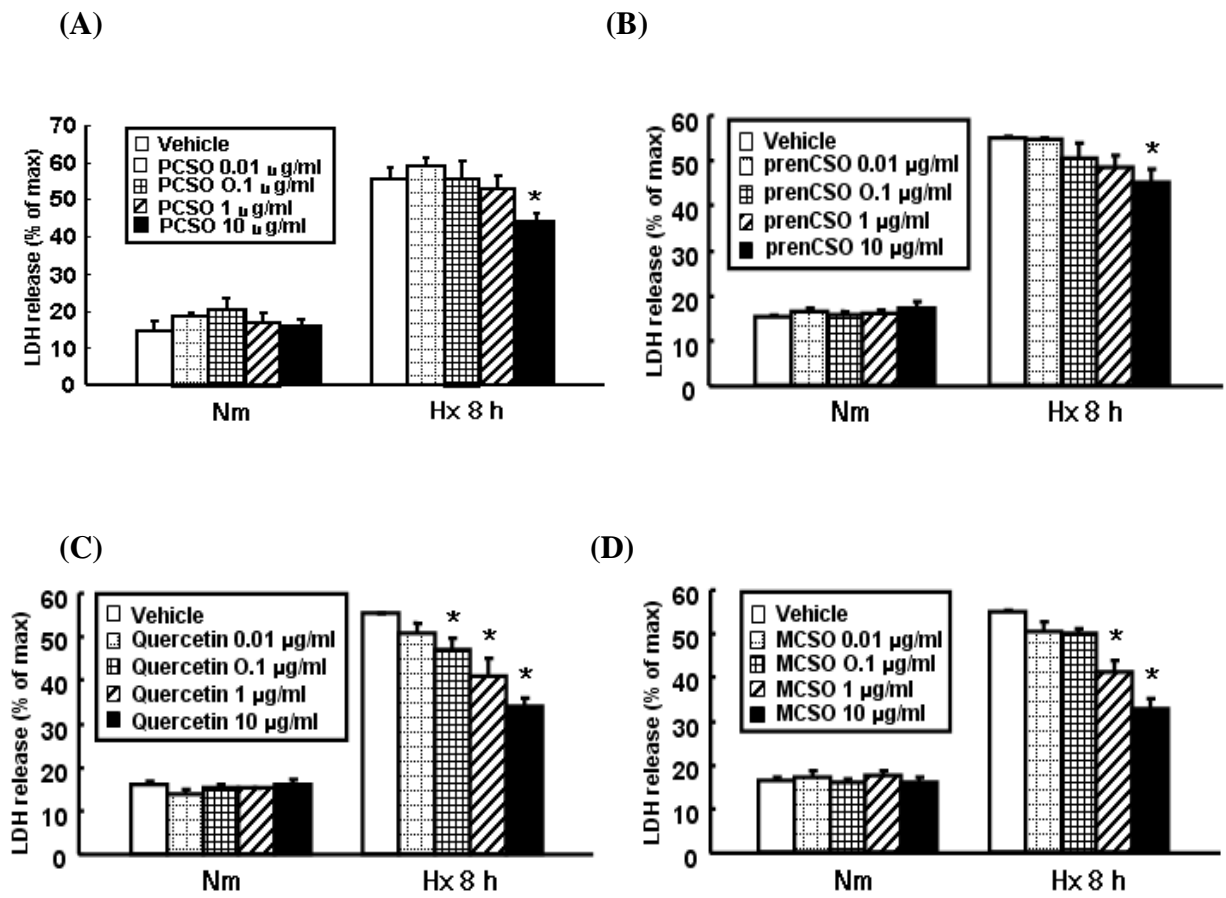


**Fig. 15. Effect of onion extract (i.v) on apoptotic cell death in rat myocardial infarct model.** (A) Representative photomicrographs of ventricular tissue stained by TUNEL method. (B) Quantitative analysis of TUNEL-positive cells. Percent TUNEL-positive cells were calculated by dividing the number of TUNEL-stained cells by total cell counts after 45 min ischemia/12 h reperfusion. All values are mean  $\pm$  SEM (n= 4). \*P<0.05 vs. Vehicle (Veh).

**Tab. 1. Key health component content in onion**

<b>MCSO</b>	<b>PrenCSO</b>	<b>PCSO</b>	<b>Quercetin</b>
<b>(mg/100g. fw)</b>	<b>(mg/100g. fw)</b>	<b>(mg/100g. fw)</b>	<b>(mg/100g. fw)</b>
<b>22.75±4.85</b>	<b>65.06±21.49</b>	<b>8.15±2.21</b>	<b>65.38±3.20</b>

\* Data taken from Kon Kuk University, Seoul, Korea.



**Fig. 16. Effects of onion components on LDH release during hypoxia.** H9c2 cells were treated with onion components (PCSO, prenCSO, Quercetin and MCSO) 30 min before and during hypoxia (Hx). All values are mean  $\pm$  SD (n= 4). \* P<0.05 vs. Hx-Vehicle. Nm means normoxia.

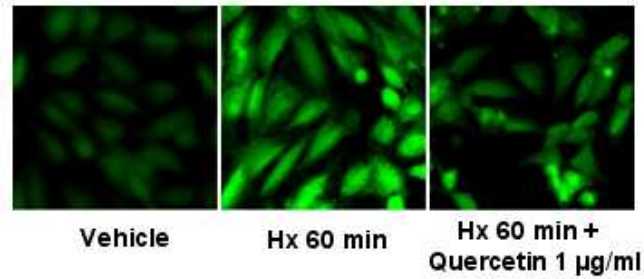
#### **N. Effect of quercetin against hypoxia-induced ROS generation**

To investigate the effect of quercetin on hypoxia induced ROS generation, we assessed the change of dichlorofluorescein (DCF-DA) fluorescence in the H9c2 cells exposed to hypoxia using confocal microscope. Fig. 18 shows the time-dependent changes in DCF-DA fluorescence. The ROS generation during hypoxia began to increase at 15 min after hypoxia and further increased to the maximum level (about 4.1 fold) at 60 min after hypoxia. The ROS generation during hypoxia 60 min was dose-dependently decreased by treatment with 0.1 and 1.0  $\mu\text{g/ml}$  quercetin.

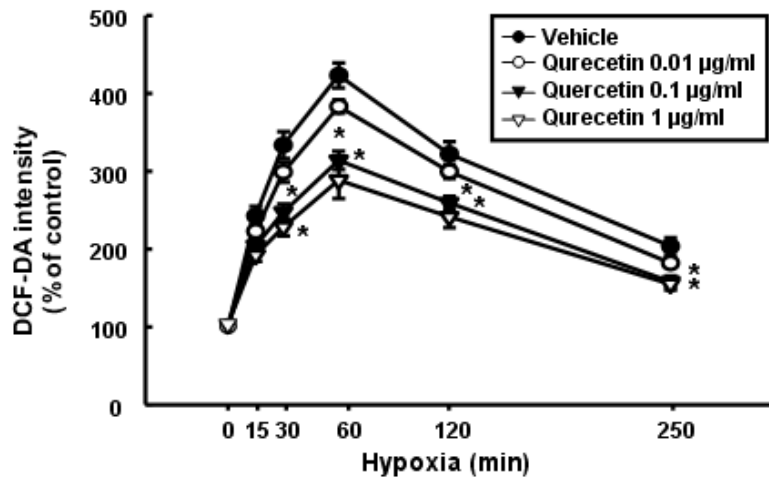
#### **O. Effect of quercetin on hypoxia-induced reduction in mitochondrial membrane potential ( $\Delta\Psi_m$ )**

To examine whether preservation of mitochondrial membrane potential ( $\Delta\Psi_m$ ) is associated with cardioprotective effect of quercetin, we assessed the change of rhodamine 123 fluorescence in the H9c2 cells exposed to hypoxia using confocal microscope. Fig. 19 shows the time-dependent changes in rhodamine 123 fluorescence. The  $\Delta\Psi_m$  was remarkably reduced by  $51.4 \pm 1.45\%$  as early as after 2 h of hypoxia and reached a maximum level ( $47.3 \pm 6.2\%$ ) after 4 h of hypoxia compared to that in normoxic control. The  $\Delta\Psi_m$  reduction after 4 h of hypoxia was completely abolished by 0.1 and 1.0  $\mu\text{g/ml}$  quercetin ( $64.6 \pm 6.00\%$  and  $64.9 \pm 5.57\%$ , respectively).

(A)

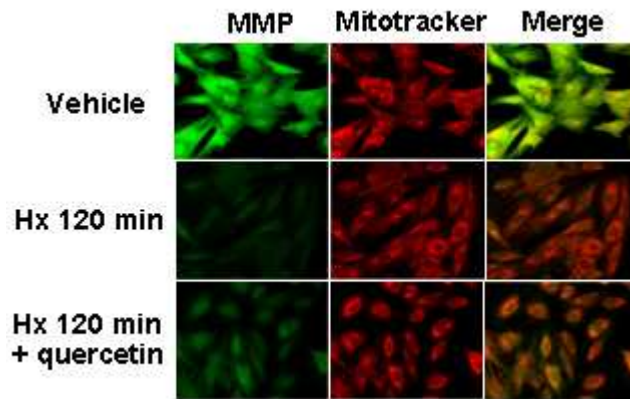


(B)

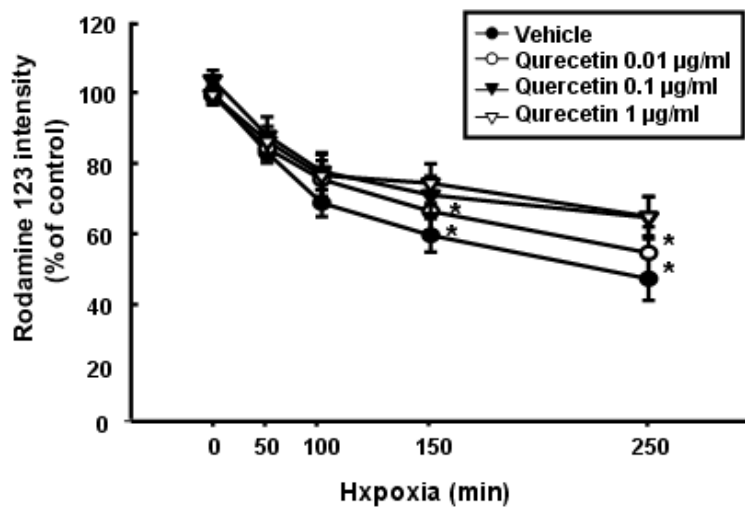


**Fig. 17. Effect of quercetin on hypoxia-induced reactive oxygen species (ROS) generation in H9c2 cells.** (A) Representative images obtained by DCF-DA confocal laser microscopy. (B) Effect of quercetin on hypoxia-induced ROS generation determined by confocal images of dichlorofluorescein (DCF-DA) staining. H9c2 cells were treated with quercetin (0.01, 0.1 and 1 µg/ml) 30 min before and during hypoxia. All data represent the mean  $\pm$  SD (n =4) \*P<0.05 vs. Hx-Vehicle. Fig. 17. shows that the protective effect of quercetin (0.01, 0.1, 1 g/ml) against hypoxia-induced cell death dose-dependently decreased LDH release. In contrast, treatment of quercetin significantly reduced LDH release.

(A)



(B)



**Fig. 18. Effect of quercetin on hypoxia-induced mitochondrial membrane potential ( $\Delta\Psi_m$ ) reduction in H9c2 cells.** (A) Representative images of rhodamine 123 intensity obtained by confocal laser microscopy. (B) Effect of quercetin on hypoxia-induced  $\Delta\Psi_m$  reduction. H9c2 cells were treated with quercetin (0.01, 0.1 and 1  $\mu\text{g/ml}$ ) 30 min before and during hypoxia. All data represent the mean  $\pm$  SD (n =4) \*P<0.05 vs. Hx-Vehicle.

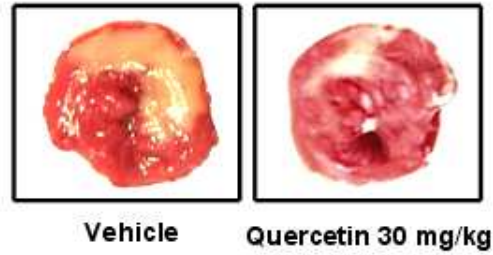
#### **P. Effect of quercetin on infarct size in rat myocardial infarction model**

To investigate the effect of quercetin on ischemia/reperfusion induced infarct size, we measured infarct size. Fig. 25 demonstrates the inhibitory effect of quercetin on the infarct size determined by TTC staining and expressed as a percentage of the infarct size/area at risk. In vehicle-treated group, ischemia (45 min) followed by reperfusion (90 min) resulted in an infarct size of  $62.8 \pm 1.96\%$  of the area at risk. Quercetin significantly reduced the myocardial infarct size in a dose dependent manner ( $55.7 \pm 5.68\%$ ,  $38.3 \pm 4.64\%$  and  $32.6 \pm 3.75\%$  at 10, 30 and 100mg/kg, respectively).

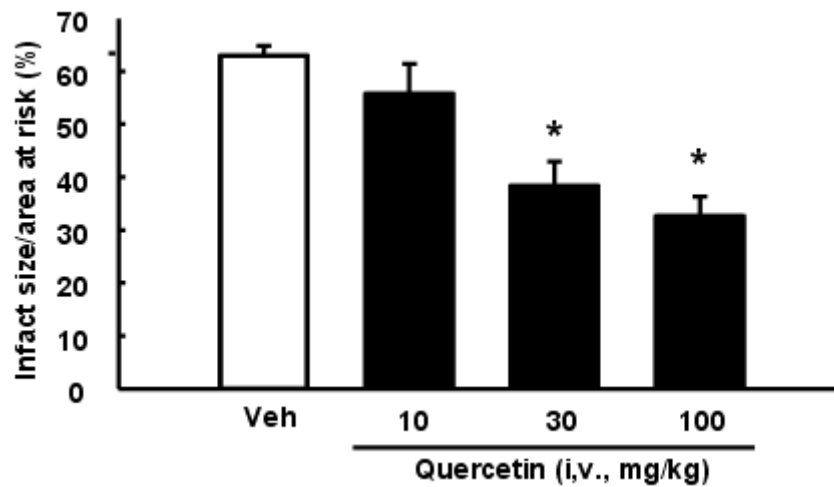
#### **Q. Effect of quercetin against ischemia-reperfusion induced apoptosis**

To investigate the effect of quercetin on ischemia/reperfusion induced apoptotic cell death, we performed TUNEL-staining. Occlusion of the left main coronary artery for a period of 45 minutes followed by 12 h of reperfusion resulted in substantial injury to the myocardium. As shown in Fig. 26, the amounts of TUNEL-positive myocytes in quercetin-treated (10, 30 and 100 mg/kg) hearts were significantly less than that in control ischemia-reperfused hearts ( $9.2 \pm 1.46\%$ ,  $7.09 \pm 1.82\%$  and  $5.17 \pm 1.61\%$  vs.  $12.5 \pm 1.69\%$ , respectively).

(A)



(B)

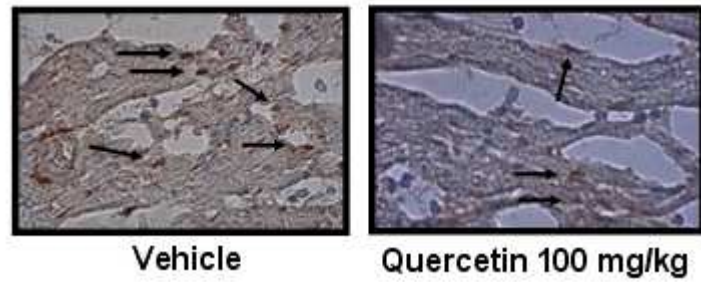


**Fig. 19. Effect of quercetin (i.v.) on infarct size in rat myocardial infarct model**

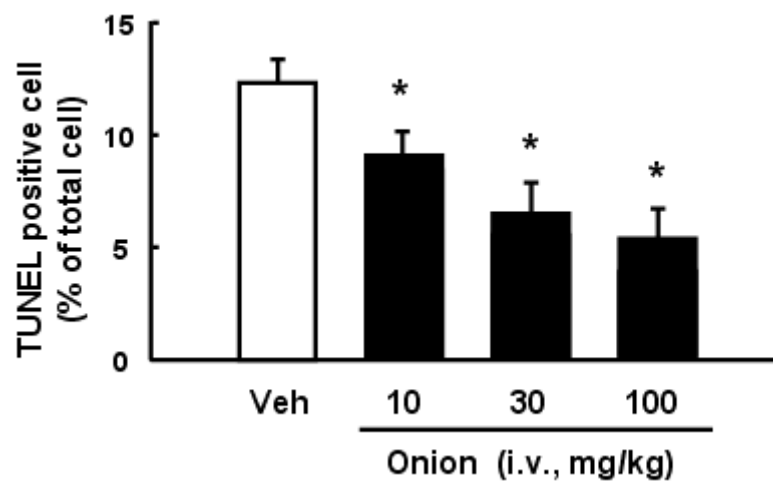
(A) Representative photomicrographs of left ventricular section. (B) Quantitative assessment of infarct size/AAR. Myocardial infarct was induced by 45 min ischemia/90 min reperfusion. quercetin (10, 30 and 100 mg/kg) were intravenously administered 20 min before occlusion. All values are mean  $\pm$  SEM (n= 8). \*p<0.05 vs. vehicle (Veh).



(A)



(B)



**Fig. 20. Effect of quercetin (i.v) on apoptotic cell death in rat myocardial infarct model.** (A) Representative photomicrographs of ventricular tissue stained by TUNEL. (B) Quantitative analysis of TUNEL-positive cells. Percent TUNEL-positive cells was calculated by dividing the number of TUNEL-stained cells by total cell counts after 45 min ischemia/12 h reperfusion. All values are mean  $\pm$  SEM (n=4). \*P<0.05 vs. Vehicle (Veh).

## V. DISCUSSION

In this study, we found the cardioprotective effect of onion extract against hypoxia-induced apoptotic death in H9c2 cells and myocardial damage in rat infarct model, by attenuating oxidative stress and maintaining mitochondrial integrity during hypoxia.

There are two basic patterns of cell death in myocardium: necrosis and apoptosis (Abbate et al., 2002; Zhao and Vinten-Johansen., 2002). Necrosis, a rapidly developed pathological processes triggered by exogenous stimuli, is primarily manifested by cell swelling or by rupture of the plasma membrane and breakdown of cell organelles with a consequent significant inflammatory response. In contrast, apoptosis, a genetically controlled progressively developed cell death under physiological or pathological conditions in response to endogenous or exogenous stimuli, is characterized by cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation without an accompanying inflammatory response (Buja., 1998; Buja., 2005) Although extensive investigation has pointed to cardiomyocyte ischemic injury evolving as the necrotic pattern of injury, more recently, apoptosis has been implicated in many forms of cardiac pathology, including myocardial ischemia.

Oxidative stress has been implicated in the pathogenesis of various cardiovascular diseases, including myocardial ischemia (Park et al., 2003; Saravanaa

& Prakash., 2004). In cardiomyocytes, ischemia/hypoxia has been demonstrated to cause oxidative stress, where decreased cytochrome oxidase activity could alter mitochondrial redox and increase generation of ROS (Chen & Lesnefsky., 2006), and the subsequent reperfusion results in an further increased concentration of ROS (Korantzopoulos et al., 2003). Recently, various studies have shown that ROS is overproduced in the pathological processes of cardiac myocytes death during hypoxia (Dutanteau et al., 1998). In the present study, we also obtained consistent data showing hypoxia-induced ROS elevation in H9c2 cells (Fig. 4).

There has been an increasing recognition that certain natural substances have the potential to reduce the detrimental effect of a number of cardiovascular risk factors. By and large, the efficacy of such biofactors lags behind pharmaceutical intervention in the amelioration of the prevalence of risk factors. Their primary use lies in the field of prevention, i.e., before the occurrence of major cardiovascular events such as myocardial infarction or strokes as caused by cerebrovascular disease. The use of natural substance has become more widespread over the past few years, driven undoubtedly by the belief that natural substances may have fewer side effects than do pharmaceuticals and by their ready availability to the public without prescriptions or visits to health providers. Onion and various forms of extracts prepared from it represent an example of such natural substances that have been claimed to possess beneficial effects for the prevention of various aspects of cardiovascular disease. This study found that onion extract, which are known to have antioxidant, not only suppressed the generation of ROS but also attenuated the number of TUNEL positive

cells and eventually protected ischemia-induced apoptosis. These results are consistent with previous studies reporting that aged garlic extract increases the level of SOD, GSH and GPx in cardiomyocyte and inhibit oxidative-stress mediated ischemia-reperfusion damage in rat heart (Banerjee et al, 2002). The variety of antioxidant phytochemicals in *Allium*, which protect against disease-causing oxidative damage (Horie et al. 1992, Ide and Lau 1997, Yamasaki & Lau. 1997), may act in single and combined fashion (Amagase et al, 1996).

Cellular redox potential is largely determined by antioxidants such as glutathione and manganese superoxide dismutase (MnSOD) located in mitochondria, which are the main organelles producing ROS and recognized as central regulators of life and death under various stresses in a variety of cells (MacLellan and Schneider, 1997). Cardiac mitochondria are highly vulnerable to ischemic injury, resulting in the precipitation of mitochondria-dependent apoptotic death pathway in a number of pathologic conditions (Capano & Crompton, 2006; Cho et al, 2004). Indeed, oxidative stress during ischemia has been demonstrated to cause mitochondrial dysfunction, which involves mitochondrial permeability transition, disruption of mitochondrial membrane potential ( $\Delta\psi_m$ ) and release of cytochrome c (Kim et al, 2005). These factors of mitochondrial dysfunction further induce the activation of downstream caspases, which in turn cause nuclear condensation, cytoplasmic fragmentation, leading to ultimate apoptotic cell death (Bialik et al, 1999). Thus, maintenance of the functional and structural integrity of mitochondria is considered as a prerequisite for successful cardioprotection. Supporting this concept, the results

from the present study has suggested that onion exerts cardioprotective effect, at least in part, through attenuation of ROS generation and maintenance of mitochondrial integrity by inhibiting  $\Delta\psi_m$  reduction, cytochrome *c* release and caspase-3 activation during hypoxia. There are controversial results about the relationship between ROS and  $\Delta\Psi_m$  reduction; one is that ROS causes  $\Delta\Psi_m$  reduction, and the other is that  $\Delta\Psi_m$  reduction causes ROS increase. The present study in hypoxia model supports the former theory, showing that  $\Delta\Psi_m$  reduction by hypoxia was attenuated by ROS scavenger trolox (Fig. 6).

Onions are rich in two chemical groups that have perceived health benefits to man. These are the flavonoids and the alk(en)yl cysteine sulphoxides (ACSOs) (Griffiths et al. 2002). Two flavonoid subgroups are found in onion, the anthocyanins, which impart a red/purple colour to some varieties and flavonols such as quercetin and its derivatives responsible for the yellow flesh and brown skins of many other varieties (Leighton et al. 1997). Among components of onion extract, quercetin makes up majority of the flavonoids of onion. Quercetin is a flavonol which functions as an oxygen radical scavenger as well as good metal chelator (Afanas'ev et al., 1989). Dak-Go et al. demonstrated that quercetin acts in many cell-free experimental systems to scavenge reactive oxygen radicals and to reduce oxidative DNA damage. In addition, quercetin is known to scavenge ROS generation in cardiomyocytes and thereby it protects against cell death in ischemia model (Duthie et al., 2004). In this study, quercetin of 1.0 and 10  $\mu\text{g/ml}$  revealed the significant protective effect against hypoxic injury in H9c2 cells. Based on the fact that quercetin decreased ROS levels,

it is can be contributed to antioxidant.

Taken together, onion extract may be useful in the prevention of ischemia-induced myocardial apoptosis due to antioxidant effect. These results will provide the mechanical basis of onion in clinical application to treat ischemic heart disease.

## VI. CONCLUSIONS

This study found that onion extract, which is known to have antioxidant, not only suppressed the generation of ROS but also attenuated cytochrome *c* release, caspase-3 activation and the number of TUNEL positive H9c2 cells. Among components of onion extract, a major component quercetin showed a remarkable protective effect against ischemic injury in vitro H9c2 cells and in vivo myocardial infarction model.

In conclusion, the results from this study suggest that onion extract protects cardiomyocyte and rat heart from hypoxia injury, contributed by attenuating oxidative stress and maintaining mitochondrial integrity during hypoxia. Therefore, the use of onion extract would be an efficient way to protect cardiomyocyte from ischemia-induced apoptotic cell death.

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## 허혈성 손상 모델에서 양파 추출물의 심근보호 효과

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심근세포의 apoptosis는 허혈성 심장질환의 병리학적 특징이며, 혈전증 또는 죽상동맥경화증에 의해 유발된다. 허혈/재관류 손상에서의 apoptosis 기작은 활성산소와 관련이 있는 것으로 보고되고 있다. 양파와 마늘 같은 *Allium*계열 식물들은 심혈관계 위험 인자를 줄여주는 효과가 있으며, 이러한 효과는 항산화 작용과 관련이 있는 것으로 알려져 있다. *Allium*계열 식물중 마늘의 허혈성 손상에 대한 심근세포 보호효과에 대해서는 많은 연구가 수행된 반면 양파의 그에 대한 효과는 거의 보고된 바가 없다.

본 연구에서는 양파 추출물이 허혈성 손상과정에서 심장보호 효과와 항산화 작용이 있는지의 여부를 H9c2 심근세포와 심근경색 동물모델에서 알아보려고 하였다. 본 연구에서는 또한 양파추출물에 함유된 구성성분들의 심근보호 효과를 관찰하였다.

H9c2 심근세포를 사용한 세포배양 모델에서, 양파추출물은 cytochrome c 방출과 caspase-3 활성을 억제하고 TUNEL 염색된 세포 숫자를 감소시킴으로써 저산소

에 의해 유발된 심근세포의 apoptosis를 감소시켰다. 또한 양파추출물은 저산소에 의한 ROS 생성을 억제하였다. 양파추출물과 항산화제로 알려진 trolox가 저산소 상태에서 cytochrome c 방출을 위한 상위 신호인 미토콘드리아 막 전위의 감소를 억제시키는 것으로 보아, 양파추출물의 anti-apoptotic 효과는 항산화작용에 의한 것으로서, 미토콘드리아 관련된 세포사멸 과정의 신호들을 억제시키기 때문으로 생각된다.

또한 양파추출물은 흰쥐를 사용한 심근경색 유도 동물모델에서도 심장보호효과를 나타냈다. 양파추출물을 흰쥐의 정맥과 구강으로 투여한 경우, 심근경색 부위의 크기를 현저하게 감소시켰고, 양파를 정맥 투여한 경우에서 TUNEL 염색된 세포의 숫자를 감소시켰다.

양파추출물의 주요 성분인 quercetin은 동물실험 모델에서 뿐만 아니라 세포 배양 모델에서도 허혈성 손상에 대한 현저한 보호효과를 나타내었고 그 양상이 양파추출물의 심근보호효과와 동일하였다.

결론적으로, 본 연구의 결과는 양파추출물의 심근세포 보호효과는 양파의 주요성분인 quercetin의 항산화 작용에 의한 것이며, 미토콘드리아 막 전위, cytochrome c 방출과 caspase-3 활성화와 같은 미토콘드리아 세포사멸 과정을 억제하기 때문인 것으로 사료된다.

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핵심어 : 양파, 허혈/재관류 손상, apoptosis, ROS, 심근세포, 항산화, 활성산소,