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Effects of *Nelumbinis Semen* on Contractile Dysfunction in Ischemic and Reperfused Rat Heart

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Nelumbinis Semen (NS), or lotus seed, is one of the most well-known traditional herbal medicines and is frequently used to treat cardiovascular symptoms in Korea. The anti-ischemic effects of NS on ischemia-induced isolated rat heart were investigated through analyses of changes in blood pressure, aortic flow, coronary flow, and cardiac output. The subjects in this study were divided into two groups: a control, untreated ischemia-induced group, and an ischemia-induced group treated with NS. There were no significant differences in perfusion pressure, aortic flow, coronary flow and cardiac output between the groups before ischemia was induced. The supply of oxygen and buffer was stopped for ten minutes to induce ischemia in isolated rat hearts, and NS was administered during ischemia induction. NS treatment significantly prevented decreases in perfusion pressure, aortic flow, coronary flow and cardiac output under ischemic conditions ($p<0.01$). In addition, the mechanism of the anti-ischemic effects of NS was also examined through quantitation of intracellular calcium content in rat neonatal cardiomyocytes. NS significantly prevented intracellular calcium increases induced by isoproterenol ($p<0.01$). These results suggest that NS has distinct anti-ischemic effects through calcium antagonism.

Key words: *Nelumbinis Semen*, Traditional herbal medicine, Anti-ischemic effect, Cardiomyocyte, Isoproterenol, Calcium antagonism

INTRODUCTION

Cardiac ischemia is a condition in which blood flow and oxygen supply to the heart muscle are insufficient. The main cause of cardiac ischemia is narrowing of the coronary arteries. When these arteries are narrowed, less blood and oxygen reach the heart muscle. Cardiac ischemia leads to coronary heart disease, angina pectoris, myocardial infarction, heart failure and, ultimately, heart attack (Shirai, 2004). Aspirin, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering agents are currently the backbone of pharmacologic therapy for this disorder (Mehta *et al.*, 2000). However, because of the adverse effects associated with these anti-ischemia drugs, the discovery and development of new anti-ischemic drugs from herbal medicines have gathered interest in attempts to minimize side effects. Numerous animal and clinical studies of various herbal medicines have been performed, and some have reported significant improvements in controlling ischemic symptoms without any noticeable adverse effects (Sun *et al.*, 2002). *Nelumbinis Semen*, or lotus seed, has been reported to both decrease blood lipids induced by a high-fat diet in rats (la Cour *et al.*, 1995) and inhibit platelet aggregation (Yu and Hu, 1997). Other potential therapeutic effects of NS extracts, such as hepato-protective effects, anti-oxidative activity (Sohn et al., 2003), and anti-arrhythmic effects (Li *et al.*, 1989a; Li *et al.*, 1989b; Li *et al.*, 1990)

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have also been previously reported. In addition, NS is known to contain various alkaloids (Wang *et al.*, 1991; Zelenski, 1977). Among these alkaloids, neferine is known for its anti-arrhythmic action (Li *et al.*, 1989a, 1989b, 1990), while nulciferine and isoquecertin are known for tranquilization (Nieto-Sampedro *et al.*, 1980) and spasmolytic actions (Lozoya *et al.*, 1994), respectively. Furthermore, we recently found that NS had distinct anti-depressant effects, in that it reduced the immobility time of rats in the forced swim (Kang *et al.*, 2005), reversed decreases of sucrose intake, and enhanced $5\text{-}HT_{1A}$ receptor binding (Jang *et al.*, 2004) and 5-HT release induced by chronic mild stress (CMS) (Kang *et al.*, 2005). To date, however, no anti-ischemia effects for NS in the heart have been reported. Therefore, in this investigation, the anti-heart ischemia effects of NS were intensively examined using an *ex vivo* Langendorff system and *in vitro* calcium imaging of neonatal cardiomyocytes.

MATERIALS AND METHODS

Preparation of *Nelumbinis Semen*

The sprayed, dried extracts of NS used in this study were purchased from Sun Ten Pharmaceutical Company (Taipei, Taiwan). The NS control used in the alkaloid comparison was purchased from the Shin-Woo Pharmacognosy Association, Seoul, Korea. One hundred grams of the dried plant were pulverized and extracted for 1 h at 100°C under reflux. The resultant solutions were filtered and the supernatants collected. The supernatants were then concentrated using an evaporator (Eyela, Japan), lyophilized with a lyophilizer (Eyela, Japan), and maintained at 4×C until use. The resulting yield of this herbal plant extract was approximately 21.3%.

Preparation of total alkaloids from NS

The standard constituents in the water extract of NS have not been reported. However, the major phytoconstituents present in other parts (excluding the seeds, but including the rhizomes) of *Nelumbo nucifera* include alkaloids like dauricine, lotusine, nuciferine, pronuciferine, liensinine, isoliensinine, roemerine, nelumbine and neferine, which are obtained through organic solvent extractions. Furthermore, neferine and liensinine have been widely recognized as having Ca^{2+}-antagonist effects. Thus, we isolated the total alkaloids from NS using organic solvents and used these isolates as standard materials in this study. Fifteen grams of each dried extract of NS (Sun Ten and control) were alkalinized with 150 mL of 5% NH_4OH and extracted with 150 mL of CH_2Cl_2 three times. The remainder of the aqueous extract was also alkalinized with 150 mL of 5% NH_4OH and extracted with 150 mL of chloroform three times. The resulting organic fractions of each extraction were merged, evaporated using an evaporator (Eyela, Japan), lyophilized with a lyophilizer (Eyela, Japan), and maintained at 4°C before use. The dried material represented total alkaloids for the Sun Ten and control NS preparations.

HPLC Analysis of total alkaloids of NS

One thousand milligrams of Sun Ten total alkaloid extract, including 31% starch, were accurately weighed, placed in test tubes, and dissolved in 10 mL of chloroform and acetonitrile (50:50 (v/v) solution) (HPLC reagent, J.T. Baker Co. Ltd, U.S.A.). This mixture was filtered with a 0.45 μm syringe filter (PVDF, Waters, U.S.A.). The control total alkaloids were used as a marker substance (standard material) for the quantitative analysis. Ten milligrams of each total control alkaloid extract was dissolved in the Sun Ten extract solution (50:50 chloroform:acetonitrile, v/v). This solution was then diluted to 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL. In order to obtain a standard HPLC chromatogram, each standard solution was also diluted to 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL in the HPLC solvent. The relationship between the concentration and peak-area was measured by the minimum square method (R^2 value). The HPLC apparatus was a Waters Breeze System (717+ Autosampler, 2487 dual λ absorbance detector, 1525 binary HPLC Pump, Waters Co., Milford, U.S.A.). Another Waters Breeze System (Ver. 5.00, Waters Co., Milford, U.S.A.) was used for data acquisition and integration. The quantity of standard total alkaloid material solution added to each herbal extract was calculated using the following formula: Amount (mg) of standard materials = {quantitative amount (mg) of standard materials × AT/AS}/n (n=3) where AT is the peak-area of the test samples containing the standard total alkaloid materials and AS is the peak-area of standard total alkaloid materials). From the results of the standard calibration curve, the R^2 values of all marker substances ranged between 0.991 and 0.999. The standard materials used for the quantitative analysis of NS were total alkaloids and their content in NS was 2.00 ± 0.85 mg/g (0.20 ± 0.08%).

Heart preparation and perfusion apparatus

Male Sprague-Dawley rats weighing 250 g to 300 g were supplied by Taconic Korea (Taconic Korea, Seoul, Korea). The rats were housed under strictly controlled pathogen-free conditions (room temperature: 23 ± 1°C, relative humidity: 50 ± 10%, light cycle: 07:00-19:00) and allowed free access to food and tap water. The rats were fed a standard rodent pellet chow and acclimatized to their environment for 2 weeks before commencement of the experiments. Next, the rats were randomly divided into 2 groups (n=10), a control ischemia-induced group and an ischemia-induced group treated with NS. The rats

were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). Heparin (1000 U/kg) was injected through a femoral vein to prevent blood coagulation. The hearts were rapidly excised and placed in ice-cold (4°C) Krebs-Henseleit (KH) bicarbonate buffer (NaCl 120.0 mM, $NaHCO_3$ 25 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.25 mM, $MgSO_4$ 1.2 mM, and glucose 11.0 mM), which immediately stopped the contractile activity of the heart. Aorta and left atrium cannulation was performed rapidly, and the hearts were perfused in Langendorff mode at a pressure of 100 cm H_2O with KH buffer. The buffer was saturated with 95% O_2/5% CO_2 at pH 7.4 and thermostatically kept at a constant temperature of 37°C. Global ischemia was achieved by clamping both the aortic and atrial lines for 10 min. In Langendorff perfusion (non-working heart model), perfusion fluid entered the heart via the aorta retrograde from the aortic reservoir located 100 cm above the heart. The aortic reservoir, which was the thermostatically maintained oxygenator, carried perfusate to the aorta at a 100 cm H_2O hydrostatic pressure, maintained with the use of a constant head device (CHD). This system maintains the function of the heart, but does not maintain circulation of perfusate to the ventricle. As such, this system is used to recover and maintain heart function for 15 min after isolation and ischemia induction. In the working heart model, the left atrium cannula and aortic cannula were open, and perfusion fluid entered the heart via the left atrium from an atrial bubble trap located 20 cm above the heart. The left ventricle ejected perfusate to the aortic bubble trap *via* the aorta and elasticity chamber (aortic pressure chamber) against a 20 cm H_2O hydrostatic pressure. This system is used to maintain heart function for 20 min before induction of heart ischemia, and to recover heart function for 60 min after ischemia surgery using the Langendorff system. The system makes it possible to compare the recovery of heart function before and after induction of heart ischemia. Aortic and coronary perfusates were not recirculated in the present study. The entire apparatus was thermostatically maintained by a water jacket and coil heat chamber. Aortic flow and coronary flow were measured by timed collection of perfusate from the aortic and pulmonary trunk cannula, respectively. Cardiac output was calculated by summing the aortic and coronary flows. Heart rate was obtained by an ECG monitoring system (S & W Medico Teknik A/S, Denmark) with three electrodes attached to the epicardium. Systolic and diastolic aortic pressures were measured throughout the working heart model perfusion periods in the aortic outflow line with a hemodynamic monitoring system (S & W Medico Teknik A/S, Denmark).

Ischemia induction of isolated-perfused rat heart

Male Sprague-Dawley rats weighing 250~300 g were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). The hearts were rapidly excised and mounted on a Langendorff apparatus (IPH-W, Labo Support, Osaka, Japan) *via* the aorta, and then perfused at a constant pressure of 65 mmHg with KH buffer. The heart was constantly warmed by a circulating water jacket at 37°C. The buffer was gassed with 95% O_2/5% CO_2 at pH 7.4. To measure left ventricular pressure, a pressure transducer was connected to the aortic cannula. Heart rate was monitored from left ventricular pressure. Coronary flow was measured by coronary flow volume (mL/min). After stabilization (non-working system) to 100 cm H_2O (100 mmHg) for 15 min *via* the aortic cannula, the perfusion pressure was reduced to 20 cm H_2O (20 mmHg) for 20 min at the LA cannula (working system), and then ischemia was induced for 10 min accompanied by NS injection for 5 min. When ischemia was started, NS extract (50 mL of 3 mg/mL NS) was dissolved in KH buffer and injected into the aortic line for 5 min to observe the effects of NS on an ischemia-induced heart with a 65 mmHg perfusion pressure. Ischemic conditions were maintained for 5 additional min. In the control group, equal volumes of KH buffer were injected into the aortic line for 5 min. Those hearts were retrograde perfused for 15 min according to the Langendorff method as described by Li *et al.* (1996) (Li *et al.,* 1996) to recover heart function. Then, the heart was perfused again through the working heart system for 60 min. The functional recovery rates between the control ischemia-induced group and ischemia-induced group treated with NS after ischemia induction were compared through changes in perfusion pressure, aortic flow, coronary flow and cardiac output to observe the anti-ischemia effect of NS.

Preparation of cardiomyocytes from neonatal rats

The hearts from 20-30 neonatal (1~3 days) Wistar rats were minced in Krebs-Ringer buffer. The tissue was subjected to five cycles of digestion in a mixture of collagenase type II and pancreatin containing 80 U/mL and 0.6 mg/mL, respectively. The cell suspension was then incubated with DNAse (0.01 mg/mlL for 10 min and filtered through a nylon mesh. The cell suspension was centrifuged at 350×g for 40 min in a discontinuous Percoll gradient to obtain a cardiomyocyte-enriched fraction. The fraction was then washed twice in DMEM by centrifugation at 200xg for 10 min. The cells were plated in a Petri dish for one hour to allow the remaining fibroblasts to attach. Unattached cardiomyocytes were then plated in 96-well plates (15,000 per well) covered with gelatin in a culture medium (DMEM :M 199=4:1) supplemented with 10% horse serum, 5% fetal calf serum, antibiotics (50 mg/mL penicillin, 50 mg/mL streptomycin) and 10 mg/mL cytosine-1--D-arabinofuranoside. The rat neonatal cardiomyocyte cultures were prepared before each experiment, and all of the experiments

were performed 48 h after culture preparation when the cardiomyocytes were contracting spontaneously. The growth medium was then replaced every 3 days.

Intracellular Ca^{2+} imaging

Cells were incubated for 40-60 min at room temperature with 5 μM fura-2/AM (Molecular Probes, Eugene, OR) and 0.001% pluronic F-127 in a Hepes-buffered solution composed of (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, and 10 glucose, with the pH adjusted to 7.4 with NaOH. Cells were illuminated with a xenon arc lamp, and excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments, CA). Emitted fluorescence was reflected through a 515-nm long-pass filter to a frame transfer cooled CCD camera, then the ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA). The inhibitory effects of NS were expressed as a percentage of the maximal value of intracellular Ca^{2+} induced by 1 nM isoproterenol initially administered to each cell. In all cases, each experiment was repeated four to five times.

Statistical analysis

The results are presented as the mean ± SEM. Statistical significance was compared between the treatment and control groups by Student's *t*-test. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Determination of maximal effective dose of NS in ischemia-induced isolated rat heart

The maximum effective amount of NS in ischemia-induced isolated rat heart was assessed by measuring cardiac output, the direct parameter of heart pump function, with and without NS treatment after induction of ischemia; increasing NS doses were administered, ranging from 0.1 mg/mL to 30 mg/mL. As seen in Fig. 1, there was no difference between groups with and without 3 mg/mL of NS treatment under pre-ischemic conditions [89.5±3.2 (98%) vs. 91.4±3.3 (100%)]. This result suggests that NS itself does not influence cardiac output under normal conditions. Although the recoveries for cardiac output after ischemia with treatments of 10.0 mg/mL and 30 mg/mL NS were not significantly different (65.8 ± 4.0 ml/min and 63.3 ± 4.9 mL/min, respectively, $p > 0.05$), the recovery for cardiac output after ischemia with 10.0 mg/mL NS appears slightly higher than that of 30 mg/mL. Thus, the maximum recovery effect for cardiac output after ischemia was apparently obtained with 10.0 mg/mL

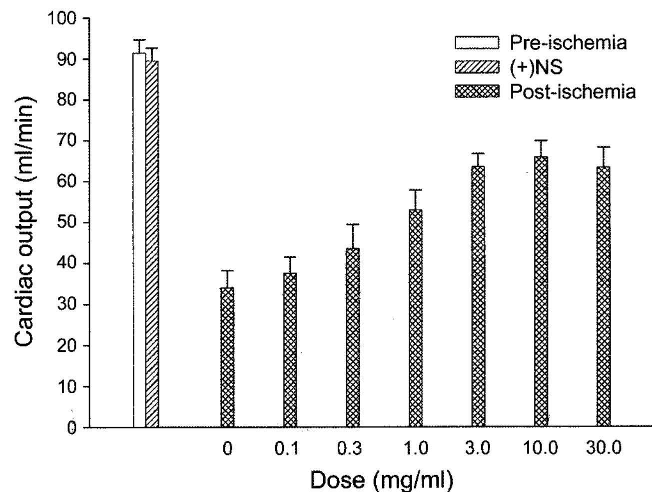

Fig. 1. Determination of minimum NS dose that results in maximum anti-ischemic effect. Perfusion pressure was measured throughout the working heart model perfusion periods with a hemodynamic monitoring system in the aortic outflow line in both groups to detect the maximal anti-ischemia effect according to NS dose (0-30 mg/mL). White histograms represent the mean ± SEM from ten rats per control group without any treatment under pre-ischemia conditions. Striped histograms represent the mean ± SEM from ten rats treated with 3.0 mg/mL NS under pre-ischemia conditions. Web histograms represent the mean ± SEM from ten rats per NS treatment group under post-ischemia conditions according to NS dose (0-30 mg/mL).

of NS. However, 10 mg/mL of NS, the concentration of apparent maximum recovery effect for cardiac output, is a very high concentration for practical applications. Furthermore, the recoveries for cardiac output after ischemia with treatment of 3 mg/mL and 10.0 mg/mL NS were not significantly different (63.5 ± 3.2 mL/min and 65.8 ± 4.0 mL/min, respectively, $p > 0.05$). Thus, 3.0 mg/mL was determined to be the appropriate NS dose to optimize the anti-ischemic effect on ischemia-induced isolated rat heart.

Heart rate in ischemia-induced isolated rat heart

Since heart rate does not significantly change under ischemic conditions (Galagudza *et al.,* 2004; Yu *et al.,* 2001), the heart rate of ischemia-induced isolated rat heart was assessed. As shown in Table I, the heart rates under pre-ischemic and post-ischemic conditions were not significantly different [275.7 ± 25.3 (100%) vs. 263.6 ±

Table I. Heart Rate in Ischemia-induced Isolated Rat Heart

Group	Pre-ischemia (beats/min) 15 min	Post-ischemia (beats/min)		
		10 min	30 min	60 min
Control	275.7±25.3 (100%)	268.7±12.5 (97.5%)	262.9±14.1 (95.4%)	263.6±16.8 (95.6%)
NS Treatment	268.5±18.7 (100%)	253.7±15.8 (94.5%)	258.7±19.2 (96.4%)	255.8±16.1 (95.3%)

16.8 (95.6%)]. Also, the heart rates of the control and NS treatment groups under post-ischemic conditions were not significantly different [263.6 ± 16.8 (95.6%) vs. 255.8 ± 16.1 (95.3%)]. These results indicate that heart rate does not change in ischemia-induced isolated rat heart regardless of NS treatment.

Overall anti-ischemic effects of NS on ischemia-induced isolated rat heart

The degree of ischemic injury was assessed by measuring the extent of perfusion pressure, aortic flow, coronary flow, and cardiac output, all of which are basic assessments of cardiac function. Induction of ischemia substantially decreased all four parameters to an average of 64.4±2.5%, 49.0±2.3%, 61.7±1.8%, and 52.3±2.3%, respectively (with 100% being the pre-ischemic value, Table II and Fig. 2). However, NS treatment recovered these decreases to an average of 81.1±2.7%, 64.9±2.8%, 92.2 ±1.9%, and 71.5±3.2%, respectively, compared to pre-ischemic conditions ($p < 0.01$, Table II and Fig. 2). These recovery rates correspond to average increases of 26% (perfusion pressure), 32% (aortic flow), 49% (coronary flow), and 37% (cardiac output) compared to control under post-ischemic conditions ($p < 0.01$, Fig. 2). These results indicate that NS treatment significantly recovered heart dysfunction induced by ischemia.

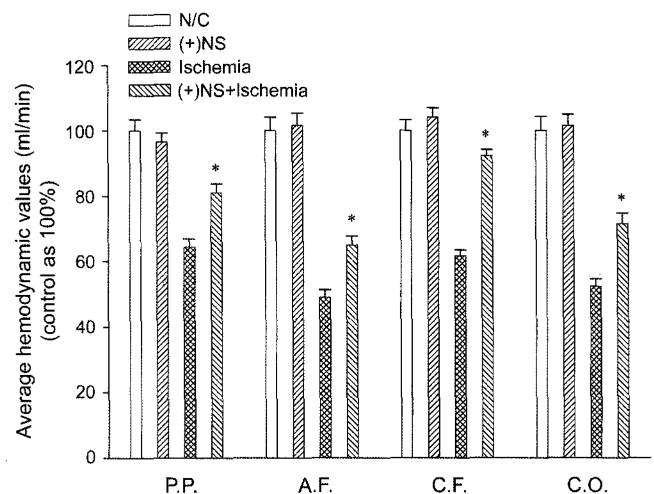

Fig. 2. Overall anti-ischemic effects of NS in ischemia-induced isolated rat heart. Perfusion pressure (PP), aortic flow (AF), coronary flow (CF), and cardiac output (CO) were measured by timed collection of perfusate from the aortic and pulmonary trunk cannula of both groups to detect anti-ischemic effects. Each histogram represents the mean ± SEM from ten rats per group: the control group under pre-ischemic conditions (first histogram, N/C), the NS treatment group (second histogram, (+) NS) under pre-ischemic conditions, the control group under post-ischemic conditions (third histogram, Ischemia), and the NS treatment group (fourth histogram, (+) NS+Ischemia) under post-ischemic conditions. *Significantly different from control group ($p < 0.01$) based on Student's *t*-test.

Recovery effect of NS on decreased perfusion pressure and aortic flow of ischemia-induced isolated rat heart

Perfusion pressure was substantially decreased by ischemia induction to an average of 64.4±2.5% of control under pre-ischemic conditions (Table II and Fig. 2). However, such decreases were recovered by NS treatment to an average of 81.1±2.7% of control before ischemia was induced ($p < 0.01$, Table II and Fig. 2). These anti-ischemic effects of NS on perfusion pressure (mmHg) were continuously observed for 10 to 60 min during the post-ischemic period (Fig. 3). However, NS had no effect on perfusion pressure under normal conditions during the experimental testing periods (control vs. NS, *p*

Table II. Overall Anti-ischemic Effects of NS on Ischemia-induced Isolated Rat Heart

Time (min)	Control				NS Treatment			
	P.P	A.F	C.F	C.O	P.P	A.F	C.F	C.O
Pre-ischemia								
5	93.7±1.0	70.3±1.2	22.9±0.8	92.2±1.5	93.9±1.8	69.7±3.2	22.5±0.5	92.2±3.2
10	93.5±0.9	67 ±1.3	23.3±0.8	90.3±1.5	93.4±1.7	68.0±3.2	23.3±0.3	91.3±3.1
15	93.8±1.1	66.7±0.9	22 ±1.0	88.7±1.3	93 ±1.8	70 ±2.0	22.5±0.6	92.5±2.0
20	92.3±1.2	66.2±1.2	22.8±0.4	89.0±1.3	92.7±1.7	68.0±1.2	22 ±0.7	90 ±1.1
Post-ischemia								
10	64.3±1.1	34.7±1.0	13.8±0.5	48.5±1.1	76.7*±2.0	40 ±3.6	18.3*±1.0	58.3*±3.6
20	63.7±1.2	34.9±1.2	13.9±0.8	48.8±1.3	75.3*±1.8	44.01*±2.1	19.0*±1.5	63.0*±2.0
30	59 ±1.3	32.4±1.4	12.7±0.7	45.1±1.6	74.5*±2.1	44.56*±2.3	20.0*±1.3	64.6*±2.3
40	58.6±1.5	31.7±1.1	13.4±1.1	45.1±1.7	73.9*±2.2	44.67*±2.4	21.0*±0.7	65.7*±2.4
50	54.1±1.1	32.4±1.4	12.9±0.7	45.3±1.6	72.9*±2.1	43.7*±2.4	20.1*±0.7	63.8*±2.4
60	51.6±1.0	31.7±1.6	13.8±0.9	45.5±1.6	73.1*±2.2	45.0*±2.5	20.7*±1.5	65.7*±2.5

* Significantly different from control group (p<0.01) based on Student's *t*-test

BP indicates blood pressure, AF aortic flow, CF coronary flow and CO cardiac output

> 0.05, Fig. 3). Taken together, these results suggest that NS does not influence perfusion pressure under normal conditions, but does recover decreased perfusion pressure induced by ischemia specifically.

Recovery effect of NS on decreased coronary flow in ischemia-induced isolated rat heart

Similarly, NS treatment successfully recovered the aortic flow reduced by ischemia to 64.9±2.8% of the control value ($p < 0.01$, Table II and Fig. 2). In the working heart model, NS treatment continuously recovered the decreases in aortic flow for 20 to 60 min after ischemia was induced (Fig. 4).

Induction of ischemia elicits a substantial decrease in coronary flow to 61.7±1.8% compared to control (Table II and Fig. 2). However, NS treatment dramatically recovered coronary flow to 92.2±1.9% of control values under pre-ischemic conditions ($p < 0.01$, Table II and Fig. 2). Such recovery continued from 10 min to 60 min in the working heart model after ischemia was induced (Fig. 5).

Recovery effect of NS on decreased cardiac output in ischemia-induced isolated rat heart

Cardiac output was substantially decreased by induction of ischemia to an average of 52.3±2.3% of control (Table II and Fig. 2). However, such decreases were

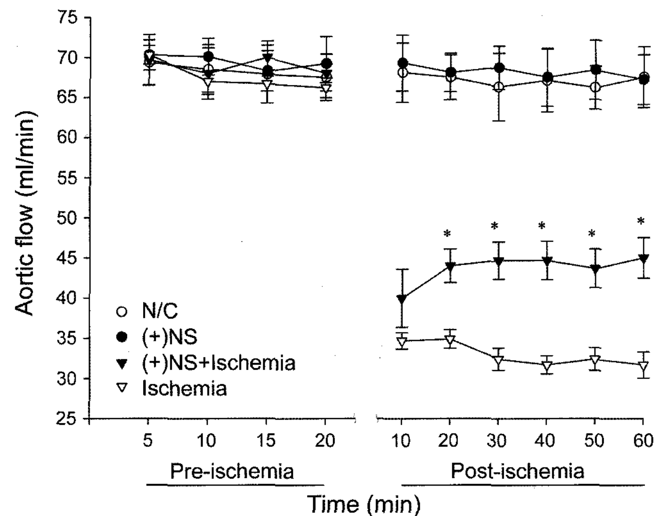

Fig. 4. Recovery effect of NS on the decreased aortic flow (AF) in ischemia-induced isolated rat heart. Aortic flow (AF) was measured by timed collection of perfusate from the aortic and pulmonary trunk cannula in the control and NS treatment groups to detect anti-ischemic effects. Each symbol represents the mean ± SEM from ten rats per group: (○) the control group under normal conditions, (●) the NS treatment group under normal conditions, (▽) the control group under ischemic conditions, and (▼) the NS treatment group under ischemic conditions. *Significantly different from the control group without any treatment under ischemic conditions ($p < 0.01$) based on Student's *t*-test.

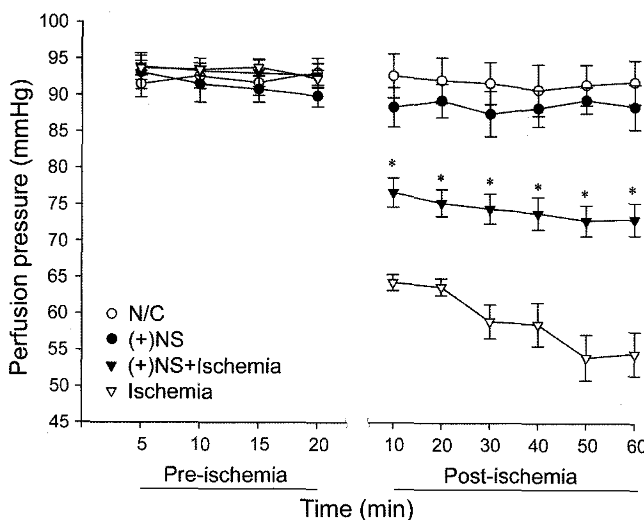

Fig. 3. Recovery effect of NS on decreased perfusion pressure (PP) of ischemia-induced isolated rat heart. Perfusion pressure was measured throughout the working heart model perfusion periods in the aortic outflow line with a hemodynamic monitoring system in the control and NS treatment groups to detect anti-ischemic effects. Each symbol represents the mean ± SEM from ten rats per group: (○) the control group under normal conditions, (●) the NS treatment group under normal conditions, (▽) the control group under ischemic conditions, and (▼) the NS treatment group under ischemic conditions. *Significantly different from the control group under ischemic conditions ($p < 0.01$) based on Student's *t*-test.

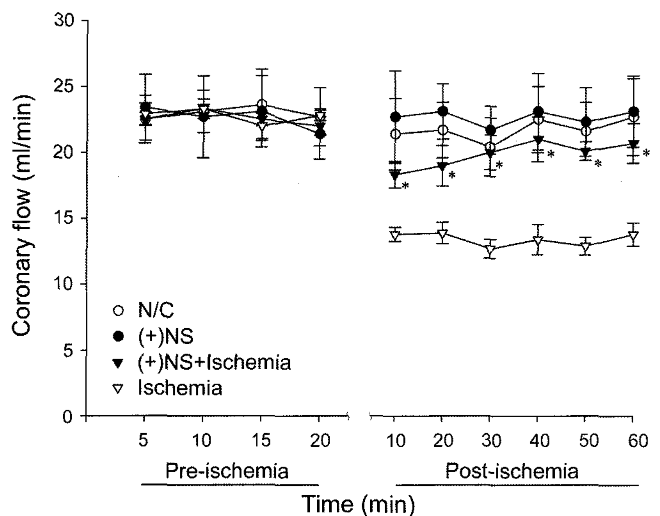

Fig. 5. Recovery effect of NS on decreased coronary flow (CF) in ischemia-induced isolated rat heart. Coronary flow (CF) was measured by timed collection of perfusate from the aortic and pulmonary trunk cannula in the control and NS treatment groups to detect anti-ischemic effects. Each symbol represents the mean ± SEM from ten rats per group: (○) the control group under normal conditions, (●) the NS treatment group under normal conditions, (▽) the control group under ischemic conditions, and (▼) the NS treatment group under ischemic conditions. *Significantly different from the control group without any treatment under ischemic conditions ($p < 0.01$) based on Student's *t*-test.

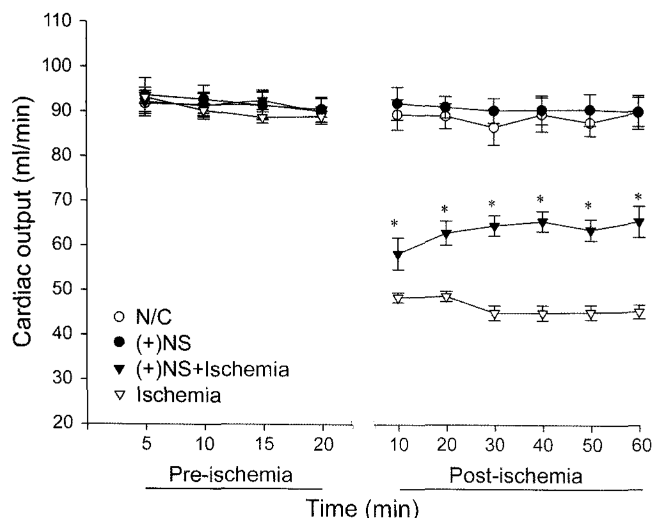

Fig. 6. Recovery effect of NS on decreased cardiac output (CO) in ischemia-induced isolated rat heart. Cardiac output (CO) was calculated by summing the aortic and coronary flows (CO=CF+AF). Each symbol represents the mean ± SEM from ten rats per group: (○) the control group under normal conditions, (●) the NS treatment group under normal conditions, (▽) the control group under ischemic conditions, and (▼) the NS treatment group under ischemic conditions. *Significantly different from the control group without any treatment under ischemic conditions ($p < 0.01$) based on Student's t-test.

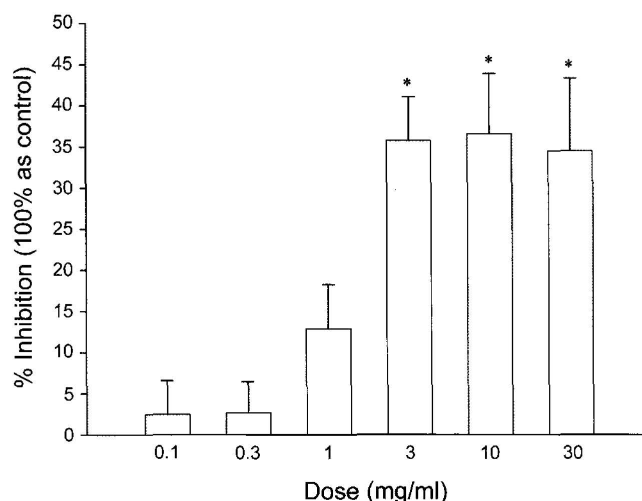

Fig. 7. Inhibitory effect of NS on isoproterenol (ISO)-mediated $[Ca^{2+}]_i$ increases in rat neonatal cardiomyocytes. Responses evoked by 1 nM ISO were quantified according to NS dose (0-30 mg/mL) to detect the maximal anti-calcium effect. Data are expressed as percent inhibition (or percent change) compared to the control response of 100% elicited by 1 nM ISO. Each bar presents the mean ± SEM from four or five cells per group, with the NS treatment groups (0-30 mg/mL) also induced by isoproterenol to cause intracellular calcium increases. *Significantly different from the control group isoproterenol-induced intracellular calcium increases ($p<0.01$) based on Student's t-test.

recovered by NS treatment to an average of 71.5±3.2% of control under pre-ischemic conditions ($p < 0.01$, Table II and Fig. 2). Also, such decreases were significantly attenuated by NS treatment by an average of 37% of control under post-ischemic conditions ($p < 0.01$, Table II and Fig. 2). In the working heart model during the post-ischemic period, NS treatment significantly recovered decreases in cardiac output (Fig. 6).

Effects of NS on intracellular Ca^{2+} overloads induced by isoproterenol in rat neonatal cardiomyocytes

The effects of NS on intracellular Ca^{2+} overloads induced by isoproterenol, a representative b-agonist (Meng *et al.*, 2005), were assessed to probe the mechanism behind the anti-ischemic effects of NS. As seen in Fig. 7, the intracellular Ca^{2+} increase induced by 1 nM isoproterenol (ISO) was significantly prevented by NS treatment at doses of 3, 10 and 30 mg/mL NS when compared to control (35.8 ± 5.3% inhibition, 36.6 ± 7.3% inhibition and 34.5 ± 8.8% inhibition, respectively, vs. 100% in control, $p < 0.01$). This result suggests that NS promotes its anti-ischemic effect through a Ca^{2+}-blocking mechanism.

DISCUSSION

Under ischemic conditions, myocardial oxidative metabolism is suppressed, and glycolysis becomes an important source of ATP generation. The increased glycolytic rate in the face of impaired glucose oxidation leads to uncoupling of the two pathways and a buildup of lactate and H^+, a process that may continue during reperfusion. This accumulation of protons leads to downstream activation of pathways (Na^+/H^+ exchanger, Na^+/Ca^{2+} exchanger) that result in Ca^{2+} overload, impaired contractile function, and/or cell death (Asano *et al.*, 2003). It is known that NS contains various chemicals (Table III), and of these components, neferine and liensinine have been most widely recognized as having Ca^{2+} antagonist effects that could potentially prevent Ca^{2+} overload or intake by the cell. Specifically, it has been reported that neferine significantly reduces the calcium influx induced by phenylephrine, an α-$_1$ agonist in the vascular muscle (Qian, 2002), and liensinine significantly inhibited slow action potentials and slow inward currents in canine cardiac Purkinje fibers (Wang *et al.,* 1993). Reactive oxygen species and metabolites are also known to play important roles in the pathogenesis of ischemia/perfusion and anoxia/reoxygenation injury. The reduction of O_2 results in the production of superoxides, as well as hydrogen peroxide (H_2O_2). H_2O_2 is highly diffusible and induces cell damage. H_2O_2 appears to affect not only lipids, but also transmembrane proteins. The hydroxyl radical (OH) also participates in lipid hyperoxidation (Asano *et al.,* 2003). Some chemicals in NS, including isoquercitrin, quercetin and hyperoside,

Table III. The Known Components of NS

• (-)-Nornuciferine	• Liriodenine
• 4'-O-Methyl-N-methylcoclaurine	• Lotusine
• Anonaine	• Methylcorypalline
• Armepavine, · dl- Armepavine	• N-Methylasimilobine (R-form)
• Asimilobine (R-form)	• N-Methylcoclaurine (R-form)
• Dehydroanonaine	• N-Methylisococlaurine
• Dehydronuciferine	• N-Norarmepavine
• Dehydroroemerine	• Neferine
• Demethylcoclaurine · Glucoluteolin	• Nornuciferine
• Hyperoside	• Nuciferine (R-form)
• Isoliensinine	• Phytol
• Isoquercitrin	• Pronuciferine (R-form)
• Kaempferol-3-O-beta-D-glucuronide	• Quercetin-3-O-beta-D-diglucopyranoside
• Liensinine	• Quercetin-3-O-beta-D-glucuronide
• Linalool	• Quercetin
• Lirinidine	• Roemerine (R-form)
• β-sitosterol	• Rutin

This table was adapted from www.tradimed.com

are recognized as antioxidants (Table III) capable of reducing reactive oxygen species (Zou *et al.,* 2004). However, it has been reported that the amounts of those important components in NS, with the exception of neferine and liensinine, are very small (Qian, 2002; Wu *et al.*, 2004). Thus, it is thought that mainly neferine and liensinine in NS work as anti-ischemic agents. Based on the previous report and this assumption, we investigated the effect of NS on intracellular calcium overload induced by isoproterenol in cultured cardiomyocytes from neonatal rats. Our data show that NS prevented isoproterenol-induced increases in $[Ca^{2+}]_i$ (Fig. 7). These results demonstrate that NS has distinct anti-ischemic effects, and preventing calcium overload in heart myocytes may be one mechanism of action of NS. However, further studies are needed to fully define the complete molecular mechanism of NS with respect to its anti-ischemic effects.

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