Effects of *Pogostemon cablin* Blanco extract on hypoxia induced rabbit cardiomyocyte injury

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ABSTRACT

Background: Pogostemonis Herba, the dried aerial part of *Pogostemon cablin* Blanco, is a well-known materia medica in Asia that is widely used for syndrome of gastrointestinal dysfunctions. **Objective:** This study was undertaken to examine whether *Pogostemon cablin* extract (PCe) might have any beneficial effect on hypoxia induced rabbit cardiomyocyte injury. **Materials and Methods:** Isolated cardiomyocytes were divided into three groups and the changes of cell viability in cardiomyocytes of hypoxic and hypoxia/reoxygenation group were determined. The effect of PCe on reactive oxygen species (ROS) generation, intracellular formation of ROS was also measured by monitoring the 2′,7′-dichlorofluorescein fluorescence. **Results:** PCe effectively protected the cells against both the hypoxia and reoxygenation induced injury, and the protective effect of PCe is not mediated by interaction with adenosine triphosphate-sensitive K^+ channels. In the presence of PCe, production of ROS under chemical hypoxia was remarkably reduced which suggests that PCe might exert its effect as a ROS scavenger. **Conclusion:** The present study provides clear evidence for the beneficial effect of PCe on cardiomyocyte injury during hypoxia or reoxygenation following prolonged hypoxia.

Key words: Cardiomyocyte, hypoxia, *Pogostemon cablin*

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INTRODUCTION

Pogostemonis Herba, dried aerial part of *Pogostemon cablin* Blanco (*P. cablin*), is a well-known herb in Korean traditional medicine, and listed in both of the current Korean Pharmacopoeia and Chinese Pharmacopoeia.[1,2] *P. cablin* can be applied for regulating vital energy and eliminating phlegm, and is a typical prescription used in treatments of apoplexy, syncope of Qi, phlegm syncope and syncope with eating and drinking.[3,4] In clinical application, therefore, it has traditionally been applied for internal use in Korean and Chinese traditional medicine to treat common colds, diarrhea and vomiting.[1,5]

Chen *et al.* reported that Pogostemonis Herba relieved the gripping pain induced by abdominal administration of acetic acid, and the effect of decoction was more potent than oil-free decoction and volatile oil, and it explains the effective components of Pogostemonis Herba may be mainly water-soluble.[6]

We previously reported its potential of reactive oxygen species (ROS) scavenger in oxidant-induced cell death of human neuroglioma cells.[3] The importance of radical scavengers in health is well-established, and a lot of degenerative diseases are related to the oxidation of biological components induced by the ROS. But the antioxidant related effects of *P. cablin* have not well identified regardless of its widespread medical applications. This study was carried out to determine whether *Pogostemon cablin* extract (PCe) might have any beneficial effect on hypoxic cardiomyocyte injury.

Hypoxia has been proposed as an important player in the pathogenesis of fibrosis, but its significance remains unclear.[7] Myocardial infarction is a typical form of the disease, which resulted from ischemic cell injury, and left ventricular dysfunction after myocardial infarction is associated with higher risk of serious ventricular arrhythmias and sudden death.[8,9]

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Recent developments in cardiac physiology indicate that ischemic-reperfusion injury may be an avoidable consequence. Murry *et al.* documented and defined the protective effect of "ischemic preconditioning" in cardiac muscle by showing that multiple brief ischemic periods before the prolonged ischemia lessened myocardial dysfunction and infarction size after reperfusion period.[10] Tissue injury resulting from ischemia is mediated, in part, by the generation of ROS such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen.[11] Cytoprotection against free radical is provided by a multilevel defense system, which comprises antioxidants (vitamin A, C, and E and reduced glutathione) and antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. It would not be absurd to expect that some Asian herbal medicines might exert their effects as antioxidants, and it has been proven to be beneficial for the patients with cerebral or cardiovascular stroke.[12-15]

Thus, this paper is devoted to determine the beneficial effect of PCe on hypoxic cardiomyocyte and measure ROS production under chemical hypoxia that could explain medicinal properties of *P. cablin* as an effective ROS scavenger.

MATERIALS AND METHODS

Pogostemon cablin extract preparation

Dried aerial parts of *P. cablin* (also called as *Patchouly plant*), were purchased from Gwangmyeong Natural Pharmaceutical Co. (Busan, Korea), and were authenticated by one of the authors (Su-In Cho, an experienced pharmacognosist) at the School of Korean Medicine, Pusan National University, where a voucher specimen (No. 201KKH) was deposited. A volume of 40 g of Pogostemonis Herba was boiled with 1000 ml distilled water at 100°C for 3 h, and total extract was evaporated under reduced pressure to give 9.5 g.

Identification for patchoulol in *Pogostemon cablin* extract and high-performance liquid chromatography fingerprint of *Pogostemon cablin* extract

For quality assurance of PCe, quantitative analysis of patchoulol, a terpene extracted from *P. cablin*, was carried out by high-performance thin layer chromatography (HPTLC) method. The presence of patchoulol in the extract was detected using HPTLC plate. Hexane and ethyl acetate (8:2, v/v) were the developer solvents. Patchoulol standards were also applied to the HPTLC plate besides the PCe. The Rf values of the standard patchoulol and the patchoulol in the PCe were compared [Figure 1a-c]. High-performance liquid chromatography (HPLC) fingerprint analysis was performed on an Agilent 1100 series HPLC system with autosampler ultraviolet-visible detector [Figure 1d].

Isolation of rabbit cardiomyocyte

New Zealand white rabbits of either sex weighing between 1.5 and 2.5 kg were used in these studies, and all animal experiments were approved by Institutional Animal Care committee of Pusan National University and conducted in accordance with the guidelines of Pusan National University. Rabbits were anesthetized with pentobarbital sodium (30 mg/kg iv) via a marginal ear vein. After a left thoracotomy, the heart was rapidly excised and mounted on a Langendorff apparatus. The heart was perfused for 5 min with calcium-free buffer containing 90 mM NaCl, 30 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 20 mM creatine, 60 mM taurine, and 1% bovine serum albumin (BSA) and supplemented with basal medium eagle vitamin and amino acid mixture. The buffer was then recirculated with 1 mg/ml collagenase and 20 μM $CaCl_2$ for 15 min. The hearts were removed, and the left ventricle was macerated and dispersed in buffer containing 115 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 20 mM creatine, 60 mM taurine, 10 mM HEPES, and 4% BSA and supplemented with vitamin and amino acid mixture. After the cells had been filtered through nylon mesh, they were washed two to three times and then made calcium tolerant by slowly restoring calcium in the medium to 1.25 mM. All buffers were gassed with 95% O_2/5% CO_2 and maintained at 37°C.

Induction of hypoxia and reoxygenation

Cells were transferred to a respiratory chamber (YSI, USA). The buffer solution in the chamber was presaturated with 95% N_2/5% CO_2 by vigorous bubbling. After transferring cells into the respiratory chamber, the buffer was gassed again with 95% N_2/5% CO_2 and sealed tightly to avoid diffusion of oxygen. After 2 h of hypoxic incubation, cells were reoxygenated by gassing the buffer with 95% O_2/5% CO_2. With this maneuver, the oxygen concentrations measured with electrodes with oxygen-selective membrane were maintained at lower than 0.5% and above 95% during hypoxic and reoxygenation periods, respectively.

Induction of chemical hypoxia

Chemical hypoxia was induced using a combination of glucose deprivation, and the mitochondrial electron transport inhibitor antimycin A as previously described.[16]

Assay for cell viability

At the indicated time points, a 50 μl aliquot of cells was removed for determination of osmotic fragility by observing whether the cells could exclude trypan blue dye in a hypotonic (85 mOsmol) solution. Those cells unable to exclude the dye are considered to have undergone membrane failure and are, therefore, deemed nonviable.

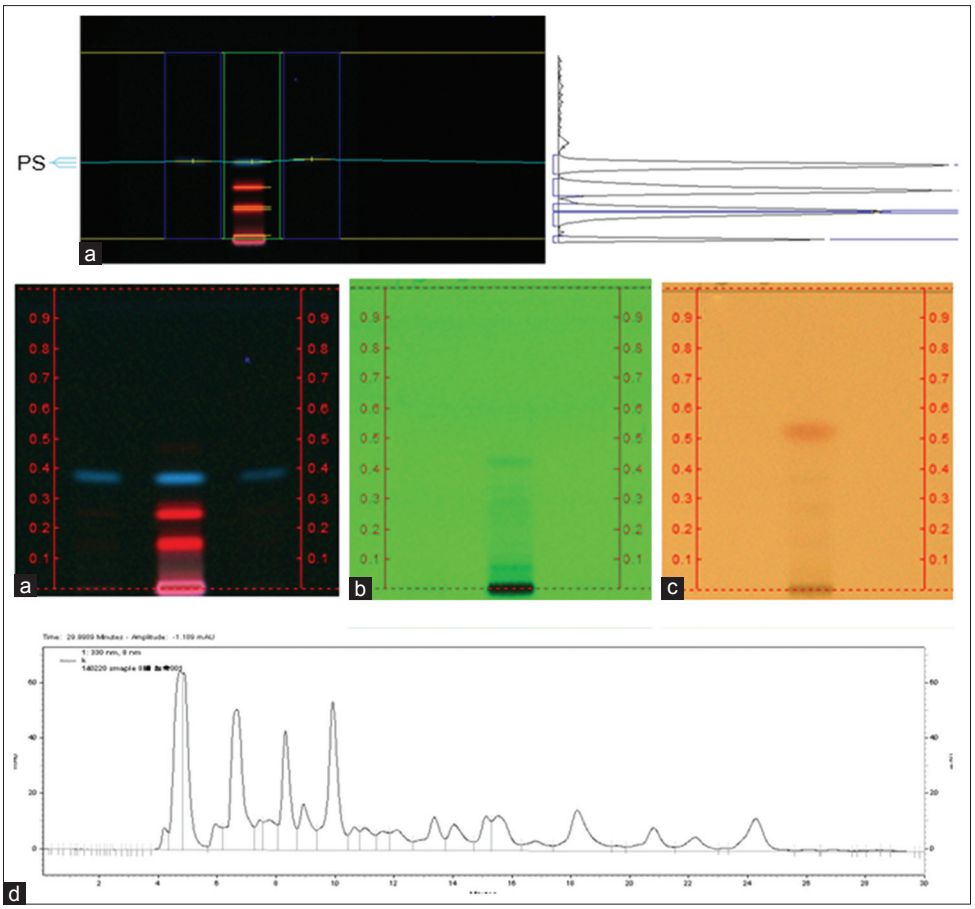

Figure 1: Images of identification and fingerprint of *Pogostemon cablin* extract (PCe). (a) in upper panel, patchoulol quantitation in PCe using high performance thin layer chromatography (silica gel F254; hexane/ethyl acetate, 8:2; 366 nm ultraviolet (UV) detected; patchoulol standard and visualizer (Camag, Swiss); (a, b) and (c) in middle panel, 366 nm UV, 254 nm UV and p-anisaldehyde sprayed under white light respectively. (d) in lower panel, high performance liquid chromatography fingerprint of PCe (column, YMC PAK pro C18 RP, 4.6 × 250 mm, 5 µm; mobile phase, 25% (1% acetic acid in acetonitrile) +75% (1% Acetic acid in water); flow rate, 0.5 ml/min; detection: 330 nm)

At least 200 cells were counted in each sample, and the percentage of stained cells was determined.

Measurement of adenosine triphosphate content

Adenosine triphosphate (ATP) levels in cells were measured by luciferin-luciferase assay.[17] Briefly, after an exposure to the experimental protocol, cells were solubilized with 500 µl of 0.5% triton X-100 and acidified with 100 µl of 0.6 M perchloric acid and placed on ice. Then cellular extract was diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4). 100 µl of 20 mg/ml luciferin-luciferase was added to 10 µl of diluted sample, and light emission was recorded for 20 s with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein concentration was determined on a portion of the cell sample and ATP content was expressed as pmoles/mg cell protein.

Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration ($[Ca^{2+}]I$) was measured in control cell suspensions and those undergoing chemical hypoxia. Cells were loaded with fura-2/AM by incubation for 45 min in the buffer containing 2 M fura-2/AM. Cells were then washed with buffer and placed in a quartz fluorescence cuvette. The fluorescence intensity was measured in a dual wavelength mode in a spectrofluorometer (SPEX1681, SPEX Co., USA). The ratio of fluorescence changes at excitation wavelengths of 340 and 380 nm with emission measured at 510 nm was recorded. The fluorescence signal was converted to $[Ca^{2+}]_i$ using the following equation as previously described.[18]

$$[Ca^{2+}]_i = (K_d)\ (b)\ [R - R_{min}]/[R_{max} - R]$$

where K_d = 225 nM (at 37°C)

$$b = I_{380,\,max}/I_{380,\,min}$$

$R = I_{340}/I_{380}$ for a given data point

$R_{min} = I_{340}/I_{380}$ for the $[Ca^{2+}]$ <10 nM endpoint

$R_{max} = I_{340}/I_{380}$ for the $[Ca^{2+}]$ >5 mM endpoint

R_{max} was determined by lysing fura-2 loaded cells with triton-X100. R_{min} was measured after chelating external Ca^{2+} with 10 mM ethylene glycol tetraacetic acid.

Measurement of reactive oxygen species production

Intracellular generation of ROS was determined by measuring 2',7'-dichlorofluorescein (DCF) fluorescence.[19] The nonfluorescent ester dye DCF diacetate (DCFH-DA) penetrates into the cells and is hydrolyzed to DCFH by the intracellular esterases. The probe is rapidly oxidized to the highly fluorescent compound DCF in the presence of cellular peroxidase and ROS such as hydrogen peroxide of fatty acid peroxides. ROS generation was measured in control cells and those undergoing chemical hypoxia. Cells were suspended in glucose-free buffer in a fluorescence cuvette. The cells were preincubated for 10 min at 37°C in a fluorescent cuvette containing 3 ml of glucose-free buffer with 20 M DCFH-DA (from a stock solution of 20 mM DCFH-DA in ethanol). After preincubation, cells were treated with antimycin A and incubated up to 120 min during which fluorescent intensity was monitored on a spectrophotometer (SPEX1681, SPEX Co., USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

Measurement of catalase activity

The catalase activity was determined by measuring the decomposition of H_2O_2 according to the method of Aebi.[20] Cells were lysed in potassium phosphate buffer containing 10 mM potassium phosphate, 10 mM $MgCl_2$ and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4 at 30°C) using a Polytron homogenizer. Cells were then centrifuged at 3,000 × *g* for 20 min at 4°C and the supernatant obtained was used for assaying the catalase activity. H_2O_2 (final 10 mM) was added to potassium phosphate buffer containing an aliquot of cellular or tissue homogenate equivalent to 100 g/ml of protein, and time-dependent decrease in absorbance at 240 nm was measured.

Measurement of Cu/Zn superoxide-dismutase activity

Cu/Zn superoxide dismutase activity was measured in cell lysate using the xanthine/xanthine oxidase and cytochrome-c reduction assay.[21] Aliquots of cell lysate was added to assay mixture containing 200 mM potassium phosphate buffer (pH 7.4), 200 mM KCl, 10 mM EDTA, 0.5 mM xanthine, 0.126 U xanthine oxidase, and 0.1 mM cytochrome c. the assay was carried out in the presence of 1.5 mM KCN. The optical density change at 550 nm was monitored at room temperature.

Measurement of glutathione peroxidase activity

Glutathione peroxidase activity was measured according to the method of Lawrence and Burk.[22] Aliquots of cell lysate was added to assay mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM NaN_3, 10 mM GSH, 1.5 mM nicotinamide adenine dinucleotide phosphate, 36 mU of glutathione reductase, and 5 mM H_2O_2. The optical density changes at 340 nm were monitored at room temperature.

Chemicals

Pinacidil and glibenclamide were obtained from Research Biochemicals International (Natick, MA, USA). DCF was purchased from Molecular Probes (Eugene, OR, USA). Other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

Data analysis

Data are presented as means ± standard error. When necessary, data were analyzed by one-way analysis of variance followed by Duncan's multiple comparison test. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Quantitative analysis of patchoulol in *Pogostemon cablin* extract and high-performance liquid chromatography fingerprint of *Pogostemon cablin* extract

Patchoulol is a terpene that is the characteristic component of the essential oil of *P. cablin*.[23] Developer was optimized by testing different solvent systems of different polarity, and the best resolution was obtained by use of hexane – ethyl acetate 8:2 (v/v), and the obtained amount of patchoulol in 1 mg/ml of PCe was 4.75 μg [Figure 1]. Documentation of the HPTLC image was performed with Visualizer and Wincat software (Camag, Swiss).

Effect of hypoxia and reoxygenation on cardiomyocyte viability

In the experiments presented in Figure 2, the time course of cardiomyocyte injury is determined. Isolated cardiomyocytes were divided into three groups. Control group was incubated in the buffer gassed with 95% O_2/5% CO_2 and hypoxic group in the buffer gassed with 95% N_2/5% CO_2 by vigorous bubbling throughout the experimental period. Hypoxia/reoxygenation group was exposed to 95% N_2/5% CO_2 for 2 h followed by reoxygenation by gassing with 95% N_2/5% CO_2. At indicated time points, aliquot of cells was removed for determination of viability by trypan blue exclusion test. In control group, cell viability was preserved throughout the experimental period, during which the percentage of viable cells was maintained at above 92%. The result of the control group was not depicted in the figure. The result in Figure 2 shows the changes of cell viability in

cardiomyocytes of hypoxic and hypoxia/reoxygenation group. When exposed to hypoxia the percentage of dead cells increased as a function of time. After 2 h of hypoxia, the percentage of dead cells reached 23.2%. Reoxygenation after 2 h of hypoxia resulted in a rapid increase in cell death. After 30 min of reoxygenation, 64.3% of cardiomyocytes were found to be nonviable. It is over two-fold the counted dead cells in the hypoxic group which was exposed to hypoxia throughout the 150 min - period. After then the dead cell count did not show any further significant change. In the following experiments, cell injury was assayed after 30-min reoxygenation following 2-h hypoxia in the hypoxia/reoxygenation group and after 150 min of hypoxia in the hypoxic group.

Effect of *Pogostemon cablin* extract on cardiomyocyte injury

It was examined first whether PCe might have any beneficial effect on cardiomyocyte injury during hypoxic period or reoxygenation period following hypoxia. Cardiomyocytes were challenged with hypoxia or hypoxia/reoxygenation in the presence of various concentrations of PCe that was added 10 min prior to the exposure to hypoxia. As depicted in Figure 3, PCe effectively protected the cells against both the hypoxia- and reoxygenation-induced injury. The effect of PCe was dose-dependent at concentrations ranging from 0.2 to 5 mg/ml. At the concentration of 2 mg/ml, it attenuated the hypoxia- and reoxygenation-induced cell death by 46.2 and 41.4%, respectively. In the experiments summarized in Figure 4, it was tested whether PCe might exert a similar beneficial effect on other types of cell injuries. For this, cardiomyocytes were exposed to chemical hypoxia or a chemical oxidant hydrogen peroxide, and the effect of PCe (2 mg/ml) was examined. The result indicates that, in addition to the protective effect against hypoxia/reoxygenation-induced injury, PCe provides a similar beneficial effect on cardiomyocyte injuries induced by chemical hypoxia or hydrogen peroxide as well.

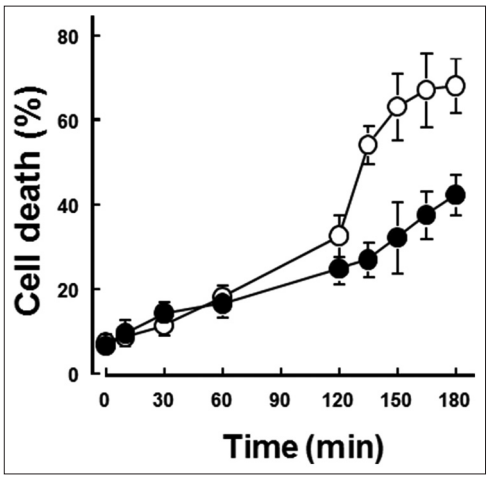

Figure 2: Effect of hypoxia and reoxygenation on cardiomyocytes viability. Isolated myocardial cells were subjected to hypoxia throughout the experimental period (closed circle) or reoxygenated after 120-min exposure to hypoxia (open circle) in respiratory chambers. At indicated time points, aliquot of cells was removed for determination of viability by observing whether the cells could exclude trypan blue dye in a hypotonic (85 mOsmol) solution. Those cells unable to exclude the dye are considered to have undergone membrane failure and are, therefore, deemed nonviable. Each point represents mean ± standard error of six determinations

During the last decade, it has been established that opening of ATP-sensitive K^+ channels during hypoxia or reoxygenation period provides a powerful protection mechanism against cardiomyocyte injuries.[24] To rule out a possible role of the ATP-sensitive K^+ channels in the PCe-induced protection against cardiomyocyte injury, effects of K^+ channel opener and blocker were examined and compared with the effect of PCe. As expected, pretreatment of cardiomyocytes with pinacidil (100 M), a well-known K^+ channel opener,[25] provided a powerful protection against cell injury induced by hypoxia/reoxygenation. The protective effect of pinacidil (100 M) was effectively blocked by glibenclamide (10 M), a blocker of the ATP-sensitive K^+ channels.[26] However, it did not affect the protective effect of PCe. These results indicate that the protective effect of PCe is not mediated by interaction with ATP-sensitive K^+ channels [Figure 5].

Effect of *Pogostemon cablin* extract on cellular adenosine triphosphate and Ca^{2+} concentrations

A decrease in intracellular ATP content and an increase in $[Ca^{2+}]I$ precedes the irreversible cell damage occurring

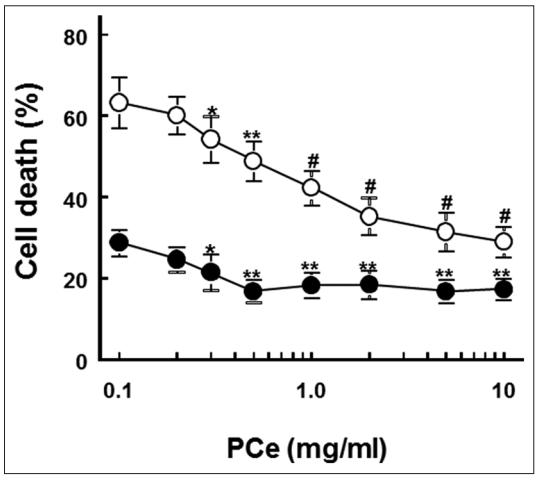

Figure 3: Concentration dependent effect of *Pogostemon cablin* extract (PCe) on cardiomyocytes viability. Isolated cardiomyocytes were subjected to 150-min hypoxia (closed circle) or 30-min reoxygenation after 120-min exposure to hypoxia (open circle) in respiratory chambers in the presence of 0.1–10 mg/ml of PCe. Each point represents mean ± standard error of four determinations. $^*P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ versus the value in the absence of PCe

upon reperfusion after prolonged periods of ischemia or hypoxia.[27,28] In the next series of experiments, it was determined whether PCe might prevent the changes of these parameters during hypoxia/reoxygenation challenge. In Figure 6, the time-dependent changes of intracellular ATP content during hypoxia/reoxygenation period in the absence and presence of PCe (2 mg/ml) is depicted. Cellular ATP content decreased to lower than 15% of its control value after 120 min of hypoxia. There was an additional decrease during the early phase of reoxygenation following hypoxia. In the presence of PCe, the decrease of cellular ATP content during hypoxia or reoxygenation period was significantly attenuated. At the present, it is difficult to make a hypoxia/reoxygenation system in which we can monitor the changes in $[Ca^{2+}]I$ simultaneously. So, instead, chemical hypoxia was induced in the fluorescence cuvette, and the changes in $[Ca^{2+}]I$ was measured by recording the fura-2 fluorescence as described in the "Materials and Methods". Upper panel in Figure 7 is a typical tracing of the fluorescence signal of the cardiomyocytes of control and chemical hypoxia. The rise in the ratio of the fluorescence signal at the excitation wavelength of 340 and 380 nm depicts the increase in intracellular Ca^{2+} concentration. In lower panel, effect of PCe (2 mg/ml) on $[Ca^{2+}]I$ in cells of control and chemical hypoxia is summarized. The result indicates that PCe effectively prevent the rise in $[Ca^{2+}]I$ induced by chemical hypoxia, whereas it did not affect the Ca^{2+} concentration in control cells.

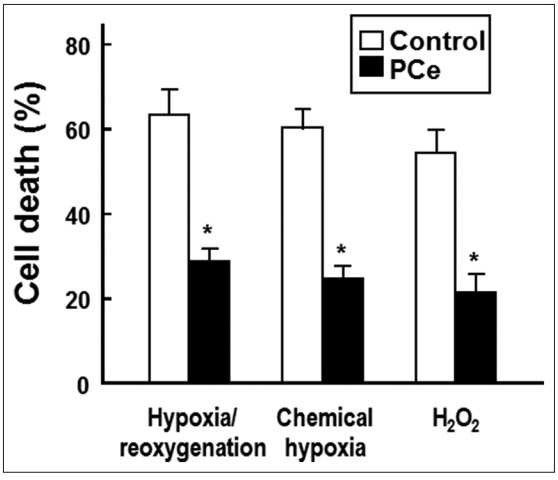

Figure 4: Effect of *Pogostemon cablin* extract (PCe) on cardiomyocytes viability exposed to different forms of oxidative stress. Isolated cardiomyocytes were subjected to 30-min reoxygenation after 120-min exposure to hypoxia, chemical hypoxia, or hydrogen peroxide (H2O2, 0.5 mM) in the absence and presence of PCe (2 mg/ml). Chemical hypoxia was induced by addition of antimycin A and glucose-deprivation. Data are presented as mean ± standard error of 4 determinations. *$P < 0.01$ versus control

Effect of *Pogostemon cablin* extract on intracellular reactive oxygen species generation and antioxidant enzyme activities

There is an increasing evidence that tissue injury resulting from ischemia/reperfusion *in vivo* or hypoxia/reoxygenation *in vitro* is mediated by intracellular generation of ROS. To elucidate the effect of PCe on ROS generation, intracellular formation of ROS was measured by monitoring the DCF fluorescence. As shown in Figure 8, chemical hypoxia induced a significant increase in the generation of ROS. In the presence of PCe (2 mg/ml), production of ROS under chemical hypoxia was remarkably reduced, suggesting that PCe might exert its effect as an effective ROS scavenger.

The effect of PCe as a ROS scavenger might result from a direct interaction with ROS or from an indirect action on innate antioxidant enzymes. To elucidate the mechanism, the effect of PCe on intracellular antioxidant enzymes was determined. PCe did not affect the activities of superoxide dismutase, catalase, and glutathione peroxidase [Table 1]. This result strongly suggests that PCe might exert its effect by acting itself as a direct scavenger of ROS.

DISCUSSION

Acute ischemia results in a loss of myocardial function. The contraction abnormality is reversible upon reperfusion when period of ischemia is short or after a more prolonged period when the myocardium is protected by collateral circulation. Necrosis, an irreversible damage, develops

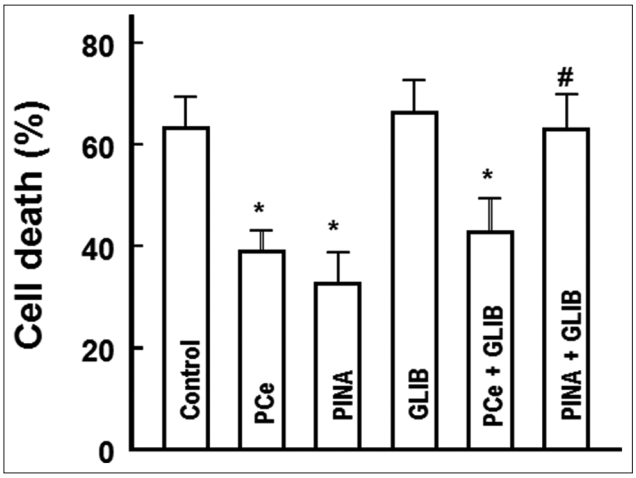

Figure 5: Effect of glibenclamide on cardiomyocytes protection by pinacidil and *Pogostemon cablin* extract (PCe). Isolated myocardial cells were subjected to 30-min reoxygenation after 120-min exposure to hypoxia in the presence of each one or combination of PCe (2 mg/ml), pinacidil (PINA, 100 M), and glibenclamide (GLIB, 10 M), and dead cells were counted. Data are presented as mean ± standard error of 5 determinations. *$P < 0.01$ versus control

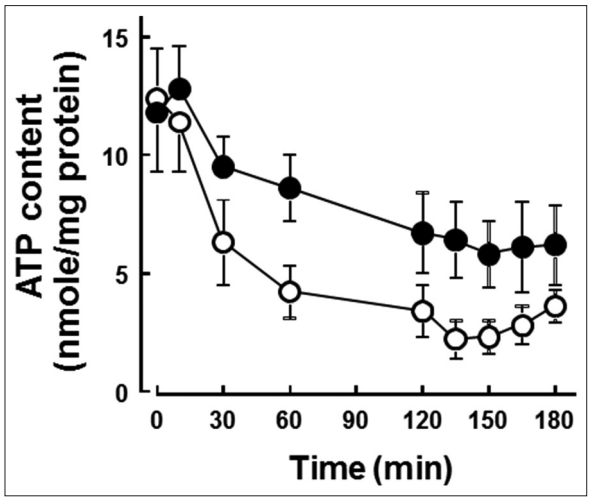

Figure 6: Effect of *Pogostemon cablin* extract (PCe) on adenosine triphosphate (ATP) content in myocardial cells subjected to hypoxia/reoxygenation. Isolated cardiomyocytes were subjected to 30-min reoxygenation after 120-min exposure to hypoxia in the absence (open circle) and the presence (closed circle) of PCe (2 mg/ml). At indicated time points, aliquot of cells was removed for determination of cellular ATP content by bioluminescence assay. Data are presented as mean ± standard error of four determinations. **P* < 0.01 versus control

Table 1: Effect of PCe (1 mg/ml) on activities of the antioxidant enzymes in the rabbit heart homogenate

	Superoxide dismutase (mU/mg protein)	Catalase (mU/mg protein)	Glutathione peroxidase (mU/mg protein)
Control	17.61±4.95	5.16±0.72	42.31±11.34
PCe	18.32±5.36	5.21±0.77	39.58±4.47

Mean±SE of 4 determinations. SE: Standard of error; PCe: *Pogostemon cablin*

when ischemia is more prolonged, of a coagulation type in the absence of reperfusion and as contraction band necrosis with reperfusion.[29] There has been an agreement that a great part of cell necrosis is occurring during the reperfusion phase.[30] The observation in this study that reoxygenation following hypoxia aggravated cardiomyocyte death is consistent with this argument [Figure 2].

For several decades there has been an active search for pharmacological agents that could render the tissue more tolerant to ischemia. It would be worthwhile to search such protective agents among the Korean traditional medicines.

The results in this study show that PCe might be an effective cytoprotectant against cardiomyocyte injury associated with ischemia or ischemia/reperfusion. It attenuated cardiomyocyte death induced by hypoxia or reoxygenation following hypoxia in a concentration-dependent manner. PCe was also effective in injuries induced by chemical hypoxia and the chemical oxidant hydrogen peroxide.

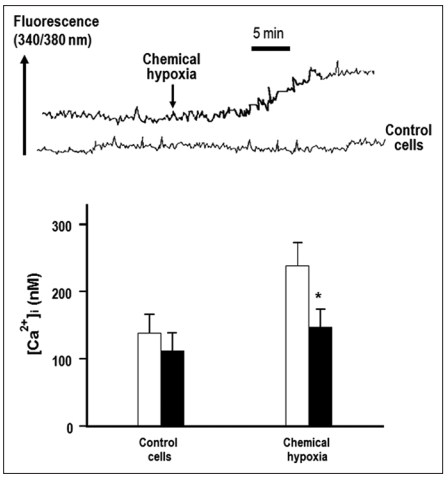

Figure 7: Effect of *Pogostemon cablin* extract (PCe) on intracellular $Ca2^+$ concentration in cardiomyocytes subjected to chemical hypoxia. Isolated cardiomyocytes were subjected to chemical hypoxia in a fluorescence cuvette in the absence (white bars) and the presence of PCe (black bars, 2 mg/ml). The ratio of fluorescence changes at excitation wavelengths of 340 and 380 nm with emission measured at 510 nm was recorded. Upper panel represents a typical tracing of the fluorescence signal. In lower panel, changes in fluorescence signal were converted to $Ca2^+$ concentrations and presented as mean ± standard error of four determinations

One of great advances in pathophysiology of ischemic heart in the recent decades is the establishment of the concept "ischemic preconditioning" by Murry *et al*.[10] They described an interesting experimental finding that brief ischemic periods before the prolonged ischemia lessened myocardial dysfunction and infarction size after reperfusion period. The nature of the end effectors responsible for ischemic protection has so far proved elusive. However, opening of ATP-sensitive K^+ channels or changes in the myocyte's actin cytoskeleton have been proposed as candidates. The ATP-sensitive K^+ channel blockers glibenclamide and 5-hydroxydecanoate have been reported to block ischemic preconditioning in a variety of species, including rabbits,[31] dogs,[32] pigs[33,34] and rats.[33] Preconditioning in human tissue also appears to involve ATP-sensitive K^+ channel because glibenclamide could block the effects of ischemic preconditioning and protein kinase C activation in human atrial trabeculae.[35]

Conversely, openers of ATP-sensitive K^+ channel are cardioprotective. Role of ATP-sensitive K^+ channel in cardioprotection is also evident in this study. Pretreatment of cardiomyocytes with pinacidil, a well-known K^+ channel opener,[18] provided a powerful protection against cardiomyocyte injury induced by hypoxia/reoxygenation. The protective effect of pinacidil was effectively blocked by glibenclamide, a blocker of the ATP-sensitive K^+ channels.[26] It was examined in this study whether

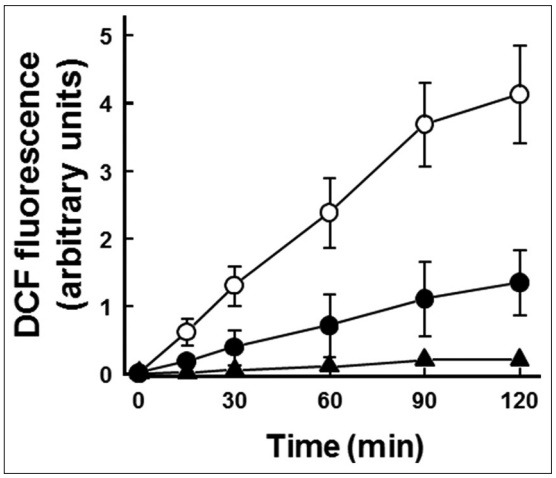

Figure 8: Effect of *Pogostemon cablin* extract (PCe) on the generation of intracellular reactive oxygen species during chemical hypoxia. 2',7'-dichlorofluorescein fluorescence were monitored in control cells (closed triangle) and in cardiomyocytes subjected to chemical hypoxia in the absence (open circle) and presence of PCe (closed circle, 2 mg/ml). Data are presented as mean ± standard error of four determinations

PCe-induced protection against cardiomyocyte injury might be due to the interaction with ATP-sensitive K^+ channels. The result indicates that the protective effect of PCe, is not mediated by interaction with the ATP-sensitive K^+ channels because glibenclamide did not affect the PCe-induced protection. The results in Figures 6 and 7 suggests that the protective effect of PCe is associated with its effect preventing the changes in intracellular ATP and Ca^{2+} concentration during hypoxia or reoxygenation following hypoxia. Cellular ATP and Ca^{2+} are parameters that are sensitive to the state of cellular oxygen supply and metabolism. The myocardial oxygen stores are consumed rapidly after cessation of blood flow. As a consequence, mitochondrial production of ATP decreases rapidly. With ATP depletion, contractility ceases, manifested first as diastolic dysfunction and within seconds, as systolic failure into a passive state. Irreversible cell damage occurring upon reperfusion after prolonged periods of ischemia or hypoxia is known to be associated with the rise in intracellular Ca^{2+}.[27,28] This rise in intracellular Ca^{2+} occurs as a result of an increase in cellular Na^+ concentration and is probably mediated by reverse mode Na^+/Ca^{2+} exchange.[36] The exact mechanism by which PCe prevent the hypoxia-induced changes in cellular ATP and Ca^{2+} was not deciphered in this study. However, it is without doubt that these effects might ameliorate the cardiomyocyte injury induced by hypoxia or reoxygenation following hypoxia.

Although underlying mechanism is not clarified yet, there is increasing evidence that intracellular generation of ROS plays a critical role in tissue injury resulting from ischemia/reperfusion. In the present study, the fluorescent dye DCFDA was used as a quantitative mean to assess the ROS generation. DCFDA has been used as a sensitive probe for cellular, mitochondrial, cytosolic, and microsomal production of ROS such as superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and etc., in many tissues.[19] The results in Figure 8 clearly showed that PCe is an effective scavenger of ROS generated under chemical hypoxia. This result suggests that the overall protective effects observed in this study might be associated with the role of PCe as an ROS-scavenger. The ROS-scavenging effect of PCe might result from a direct interaction with ROS or from the indirect effect by activating innate antioxidant enzymes. However, PCe did not affect the activities of superoxide dismutase, catalase, and glutathione peroxidase. These results strongly suggest that PCe might exert its effect by acting itself as a direct scavenger of ROS. In the present study, it is not clear which component of PCe is responsible for the ROS-scavenging effect. It may be a natural component (s) included in PCe or a newly formed compound (s) through a chemical reaction among the natural components of PCe. Further analytical study should be needed to elucidate this. The present study provides clear evidence for the beneficial effect of PCe on cardiomyocyte injury during hypoxia or reoxygenation following prolonged hypoxia. The action of PCe as an ROS-scavenger might underlie the mechanism.

CONCLUSIONS

This study was carried out to determine whether PCe might have any beneficial effect on hypoxic cardiomyocyte. PCe effectively protected the cells against both the hypoxia and reoxygenation induced injury. It provides a similar beneficial effect on cardiomyocyte injuries induced by chemical hypoxia or hydrogen peroxide as well, and the protective effect of PCe is not mediated by interaction with ATP-sensitive K^+ channels. In the presence of PCe, the decrease of cellular ATP content during hypoxia or reoxygenation period was significantly attenuated. PCe effectively prevents the rise in intracellullar Ca^{2+} concentration induced by chemical hypoxia. In the presence of PCe, production of ROS under chemical hypoxia was remarkably reduced, suggesting that PCe might exert its effect as an effective ROS scavenger. PCe might exert its effect by acting itself as a direct scavenger of ROS. The results in this study show that PCe might be an effective cytoprotectant against cardiomyocyte injury associated with ischemia or ischemia/reperfusion. In addition, the present study suggests that PCe might exert its effects as an effective ROS scavenger.

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