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Myocardial Protection by Protykin, A Novel Extract of *trans*-Resveratrol and Emodin

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Protykin is an all-natural, high potency standardized extract of *trans*-resveratrol (20%) and emodin (10%) derived from the dried rhizome of *Polygonum cuspidatum*. Previous studies have demonstrated free radical scavenging and anti-inflammatory activities of resveratrol. Since free radicals play a crucial role in the pathogenesis of myocardial ischemia/reperfusion injury, we examined whether Protykin could preserve the heart during ischemic arrest. Sprague–Dawley rats were divided into two groups: experimental group was gavaged Protykin (100 mg/kg body wt) dissolved in corn oil for three weeks, while the control group was gavaged corn oil alone. After three weeks, rats were sacrificed, isolated hearts perfused via working mode, were made globally ischemic for 30 min followed by 2 h of reperfusion. Left ventricular functions were continuously monitored and malonaldehyde (MDA) (presumptive marker for oxidative stress) formation were estimated. At the end of each experiment, myocardial infarct size was measured by TTC staining method. Peroxyl radical scavenging activity of Protykin was determined by examining its ability to remove peroxyl radical generated by 2,2′-azobis (2-amidinopropane) dihydrochloride, while hydroxy radical scavenging activity was tested with its ability to reduce 7-OH$^{\bullet}$–coumarin-3-carboxylic acid. The results of our study demonstrated that the Protykin group provided cardioprotection as evidenced by improved post-ischemic left ventricular functions (dp, dp/dt_{max}) and aortic flow as compared to control group. This was further supported by the reduced infarct size in the Protykin group. Formation of MDA was also reduced by Protykin treatment. *In vitro* studies demonstrated that Protykin possessed potent peroxyl and hydroxyl radical scavenging activities. The results of this study indicate that Protykin can provide cardioprotection, presumably by virtue of its potent free radical scavenging activity.

Keywords: Protykin, resveratrol, emodin, grape, red wine, antioxidant, ischemia/reperfusion, peroxyl radical, hydroxyl radical, heart, flavonoids, cardioprotection

INTRODUCTION

Consumption of red wine is becoming increasingly popular due to the intrigue created by the *French Paradox*. Despite the high fat diet and

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smoking tendencies of the population in the Southern France, there is lower incidence of coronary heart disease than might be suggested from such dietary habits.[1] This interesting observation has been attributed to the high consumption of red wine by the French. This beneficial effect has been attributed to antioxidants present in the polyphenol fraction of red wine.[2] Grapes contain a variety of antioxidants including resveratrol, catechin, epicatechin and oligomeric proanthocyanidins, among which resveratrol is mainly present in skin, while proanthocyanidin is present in the seeds of grapes.

Oxygen-derived free radicals have been implicated in the pathogenesis of diverse degenerative diseases including ischemic heart disease.[3–7] There is evidence to support that oxidative stress resulting from increased production of free radicals associated with decreased levels of antioxidants in the myocardium plays a crucial role in cardiovascular diseases such as ischemic heart disease, atherosclerosis, congestive heart failure, cardiomyopathy, hypertrophy and arrhythmias.[8] Apart from experimental studies, epidemiological relationships exist between oxidative stress and occurrence of cardiovascular diseases that include ischemic heart disease[9] and arteriosclerosis.[10]

This study was undertaken to investigate potential cardioprotective effects of a novel antioxidant, Protykin, derived from the dried rhizome of *Polygonum cuspidatum*, which contains 20% *trans*-resveratrol and 10% emodin. Post-ischemic ventricular recovery were compared between two groups of rat hearts, Protykin-fed group and control group. The results of our study demonstrated that *Protykin*-fed rat hearts were resistant to ischemia reperfusion injury. *In vitro* studies determined *Protykin* to be a potent scavenger of peroxyl and hydroxyl radicals. Since these reactive oxygen species are produced in the ischemic reperfused myocardium, the cardioprotective properties may be attributed at least in part, to the hydroxyl and peroxyl radical scavenging activities of Protykin.

MATERIALS AND METHODS

Protykin

Protykin™ was obtained from InterHealth Nutraceuticals, Inc., Concord, CA. Protykin is derived from the root of *Polygonum cuspidatuni*, an herb used in traditional Chinese medicine. It contains 20% *trans*-resveratrol and 10% emodin. All other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

Animals

All animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (publication no. NIH 85-23, revised 1985). Male Sprauge–Dawley rats weighing 275–300 g were provided with food and water *ad libitum* until the start of the experimental procedure. Twenty-four rats were randomly assigned to one of the two groups, control and Protykin-fed. Half of the rats were gavaged Protykin (100 mg/kg b.w./day for three weeks) dissolved in corn oil, while the other half were given corn oil only.

Experimental Design

Rats were anesthetized with sodium pentobarbital (80 mg/kg b.w., i.p. injection, Abbott Laboratories, North Chicago, IL), anticoagulated with heparin sodium (500 IU/kg b.w., i.v. injection, Elkins-Sinn Inc., Cherry Hill, NJ). After ensuring sufficient depth of anesthesia, median thoracotomy was performed, hearts were excised and immersed in ice-cold perfusion buffer. Aortic cannulation was performed as quickly as possible and hearts were perfused in the retrograde Langendorff mode at a constant perfusion pressure of 100 cm H_2O.[11]

The perfusion buffer used in this study consisted of a modified Krebs–Henseleit bicarbonate buffer (KHB) (in mM: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose and 1.7 $CaCl_2$, gassed with 95% O_2–5% CO_2, filtered through 5 μm filter to remove any particulate contaminants, pH 7.4) which was maintained at a constant temperature of 37°C and was gassed continuously for the entire duration of the experiment. Left atrial cannulation was then carried out. After allowing for a stabilization period of 10 min in the retrograde perfusion mode, the circuit was switched to the antegrade working mode which allows for the measurement of myocardial contractility as well as aortic and coronary flows, as described in detail in a previous paper.[11] Essentially it is a left heart preparation in which the heart is perfused at a constant preload of 17 cm H_2O (being maintained by means of a Masterflex variable speed modular pump, Cole Parmer Instrument Company, Vernon Hills, IL) and pumps against an afterload of 100 cm H_2O.

At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded and coronary effluent samples were collected for biochemical assays. Hearts were then subjected to global ischemia for 30 min followed by 2 h of reperfusion. The first 10 min of reperfusion were in the retrograde mode to allow for post-ischemic stabilization and thereafter, in the antegrade working mode to allow for assessment of functional parameters which were recorded at 30, 60 and 120 min into reperfusion.

Measurement of Ventricular Functions

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH) connected to a side arm of the aortic cannula. The signal was amplified using a Gould 6600 series signal conditioner (Gould Instrument Systems Inc., Valley View, OH) and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA). Heart rate, developed pressure (defined as the difference of the maximum systolic and diastolic aortic pressures) and the first derivative of developed pressure were all derived or calculated from the continuously obtained pressure signal. Aortic flow was measured using a calibrated flowmeter (Gilmont Instruments Inc.) and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

Infarct Size Estimation

At the end of each experiment, left ventricle was quickly excised, frozen, and then cut into transverse slices approximately 1 mm thick. The slices were weighed, thawed, and stained by incubation for 20 min at 37°C in 1% *triphenyl tetrazolium chloride* in phosphate buffer, pH 7.4. The areas of infarct (tetrazolium-negative) and risk zone (whole left ventricle) were determined by scanning the slices and computer-assisted planimetry using the NIH image 1.6.1 software.[12] Infarct and risk zone mass were then calculated by multiplying each area by the slice weight and summing the products. Infarct size was expressed as a percentage of the risk zone infarcted.

Estimation of Oxidative Stress

Malonaldehyde (MDA) was assayed as described previously[13] to monitor the development of oxidative stress during ischemia reperfusion. Coronary perfusates were collected at the time of recording baseline functional parameters and thereafter at timepoints of 3, 10, 30, and 120 min into reperfusion for the measurement of MDA. The MDA in the collected coronary perfusate samples was derivatized using 2,4-dinitrophenylhydrazine (DNPH). Two ml of perfusate was added to 0.1 ml of DNPH reagent (310 mg DNPH in 100 ml 2 N HCl, 1.56 mmol DNPH) in a 20 ml Teflon lined screw-capped test tube. Contents were vortexed and 10 ml of pentane was added prior to intermittent rocking for 30 min. The

aqueous phase was extracted three times with pentane, blown down with N_2 and reconstituted in 200 µl of acetonitrile. Aliquots of 25 µl in acetonitrile was injected onto a Beckman Ultrasphere C_{18} (3 mm) column in a Waters high-performance liquid chromatography (HPLC). The products were eluted isocratically with a mobile phase containing acetonitrile–H_2O–CH_3COOH (34 : 66 : 0.1, v/v/v) and detected at three different wavelengths of 307, 325 and 356 nm. The peak for MDA was identified by co-chromatography with a DNPH derivative of the authentic standard, peak addition, comparison of the UV patterns of absorption at the three wavelengths and by GC-MS. The amount of MDA was quantitated by performing peak area analysis using the Maxima software program (Waters, MA) and expressed in pmol/ml.

In Vitro Peroxyl Radical and Hydroxyl Radical Scavenging Assay

Peroxyl radical scavenging activity was examined by adding Protykin to a peroxyl radical generating system.[14] Peroxyl radicals were generated by dissolving 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) in phosphate buffered saline (PBS) at 37°C. Scavenging activity of Protykin was compared with Trolox (25 µg/ml), a well known peroxyl radical scavenger. Protykin (10 µg/ml) or Trolox (25 µg/ml) were added to the peroxyl radical generating system (final concentration of AAPH was 2 mM) and the decrease in fluorescence of 5- and 6-carboxyfluorescein (5-/6-CF) as a function of time (up to 1 h) was observed at excitation and emission wavelengths of 500 and 520 nm, respectively using a Perkin Elmer LS 50 B fluorescence spectrometer (Perkin Elmer, Norwalk, CT).

Aqueous solution of coumarin-3-carboxylic acid (CCA) was diluted in PBS buffer, pH 7.4 to give a final concentration of 10^{-5} M. Polymethylmethacrylate cuvettes containing 2.5 ml CCA solution were irradiated with ^{137}Cs γ-ray (dose rate 1.14 Gy/min) (MDS Nordian, Ontario, Canada) at room temperature. Upon irradiation, $OH^{\bullet}$ radical is produced which converts CCA in aqueous solution into highly fluorescent 7-hydroxy-CCA. Fluorescence was measured at room temperature with a Perkin Elmer LS 50 fluorimeter (excitation 400 nm, emission 450 nm).[15] The $OH^{\bullet}$ radical scavenger dimethyl sulfoxide (DMSO) abolished the induction of 7-$OH^{\bullet}$–CCA formation, indicating scavenging of $OH^{\bullet}$ radical. The scavenging activity of the Protykin extract was compared against 1 mM DMSO.

Statistical Analysis

All data are presented as mean ± SEM. One-way analysis of variance combined with Tukey's *post-hoc* test was used to test for difference in infarct size between groups. Analysis of variance with replication was used to test for difference in hemodynamics in any given group. When a significant group difference was detected, observations at individual time points were compared with paired *t*-tests by means of a Dunn–Sidak correction for multiple comparisons. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of Protykin on Post-Ischemic Ventricular Recovery

There were no differences in baseline function between control and Protykin-fed groups. As expected, on reperfusion, the absolute values of all functional parameters were decreased in both groups as compared to the baseline values, except in the case of aortic pressure and coronary flow which did not exhibit any change. Protykin-fed rat hearts displayed significant recovery of post-ischemic myocardial function. This was evidenced by significant differences in the developed pressure readings throughout the reperfusion period. Significant differences were observed at 120 min of reperfusion (48 ± 7.7 vs. 69.4 ± 2.8 mmHg) (Figure 1, top). The maximum first

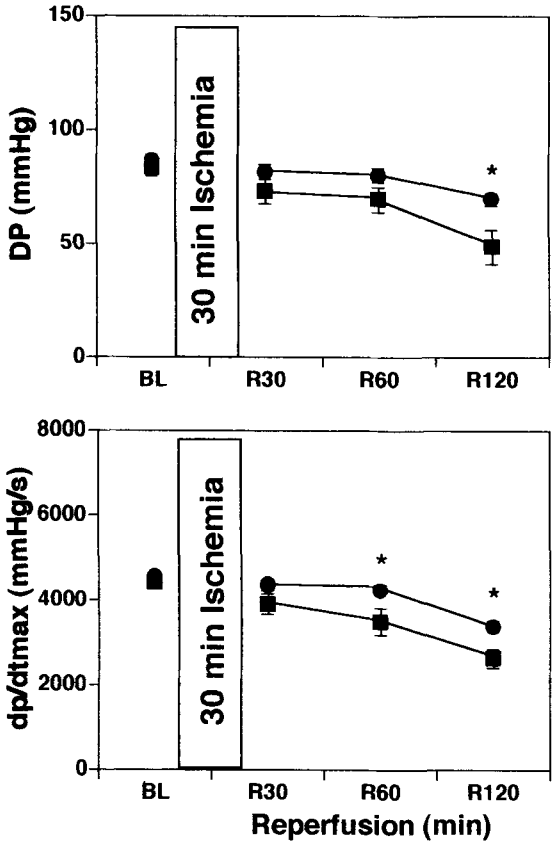

FIGURE 1 Effects of Protykin on developed pressure (top) and the maximum first derivative of developed pressure (bottom). Results are expressed as means ± SEM of six rats per group. *$p < 0.05$ compared to control. (■) control, (●) Protykin.

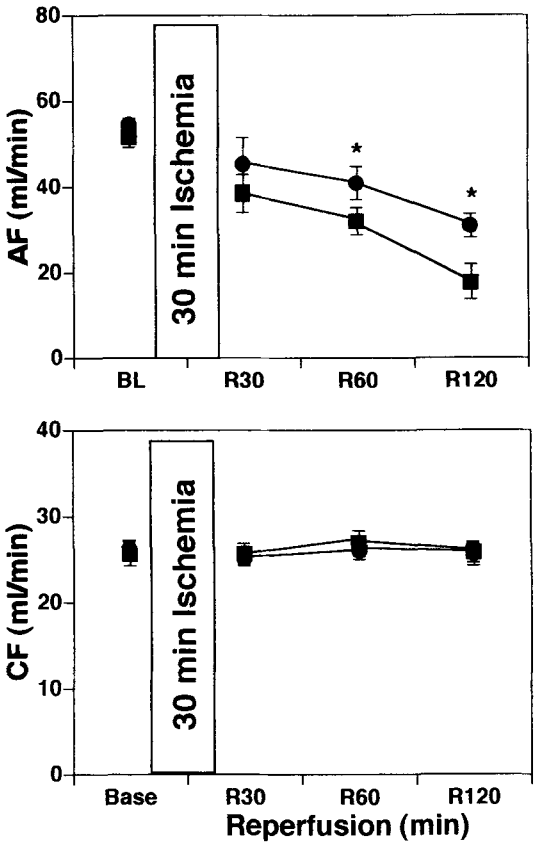

FIGURE 2 Effects of Protykin on aortic flow (top) and coronary flow (bottom). Results are expressed as means ± SEM of six rats per group. *$p < 0.05$ compared to control. (■) control, (●) Protrykin.

derivative of the developed pressure followed a similar pattern (Figure 1, bottom). Aortic flow was markedly higher in the Protykin group from R60 onwards (Figure 2, top), the difference being significant at R120 (17.6 ± 4.2 vs. 31.8 ± 1.5 ml/min). No differences were found for coronary flow (Figure 2, bottom). Heart rate did not vary between the two groups of hearts (results not shown).

Effects of Protykin on Myocardial Infarct Size

Infract mass as well as infarct size (% infarct of total volume at risk) were noticeably reduced in Protykin-fed group as compared to the control group (0.209 ± 0.017 vs. 0.158 ± 0.007 g and 24.6 ± 0.8% vs. 18.8 ± 0.4%) as shown in Figure 3.

Effects of Protykin on MDA Formation

The production of MDA is an index of the occurrence of lipid peroxidation and the development of oxidative stress. Coronary perfusate MDA levels were found to be significantly reduced in the Protykin group as compared to control group (Figure 4), the difference being especially apparent in the immediate early reperfusion period at R3 and R10. Furthermore, at no time during reperfusion were the MDA levels in Protykin-fed group significantly higher than the baseline value.

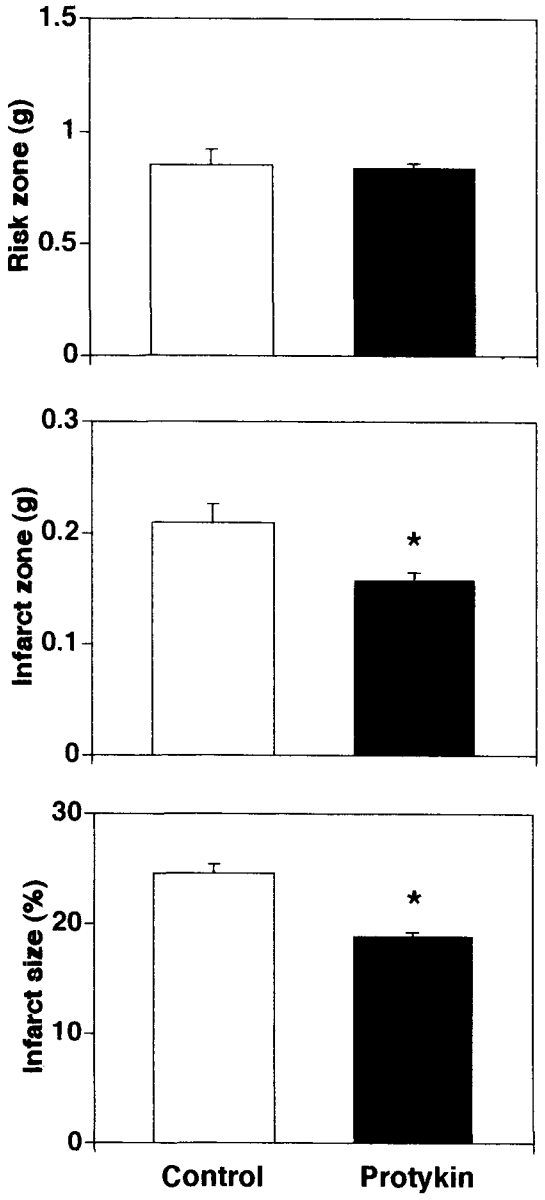

FIGURE 3 Effects of Protykin on myocardial infarction after ischemia and reperfusion. Results are expressed as means ± SEM of six rats per group. *$p < 0.05$ compared to control. (■) control, (●) Protykin.

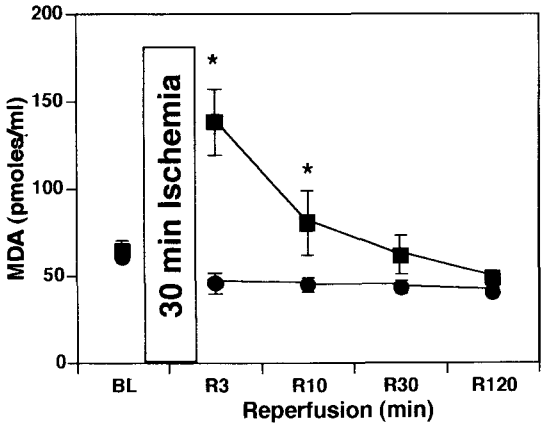

FIGURE 4 Effects of Protykin on the MDA formation during ischemia and reperfusion. Results are expressed as means ± SEM of six rats per group. *$p < 0.05$ compared to control. (■) control, (●) Protykin.

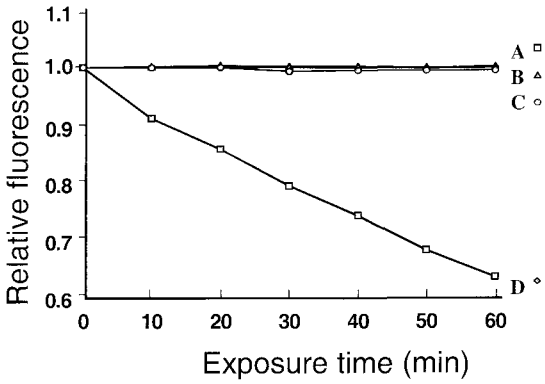

FIGURE 5 Relative peroxyl radical scavenging activity of Protykin and Trolox. A change in relative fluorescence of 5-/6-carboxyfluorescein (5-/6-CF) upon exposure to peroxyl radicals generated by the thermal decomposition of 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH) and effects of Protykin on the same. The results show the relative fluorescence obtained by CF (0.1 μM) alone (A); CF + AAPH (2 mM) (D); CF + AAPH + Trolox (25 μg/ml) (B) and CF + AAPH + Protykin (10 μg/ml) (C). Results are expressed as mean relative fluorescence of three determinations per group. The results show that both Trolox 25 μg/ml and Protykin (10 μg/ml) are equally effective in blocking the decrease of AAPH-mediated decrease in CF-fluorescence.

Peroxyl Radical and Hydroxyl Radical Scavenging Activities of Protykin

The amount of fluorescence of 5-/6-CF decreased when it was exposed for 1 h to the peroxyl radicals generated by the thermal decomposition of AAPH as shown in Figure 5. This effect of AAPH was effectively blocked by Protykin at concentrations of 10 μg/ml. This scavenging activity of

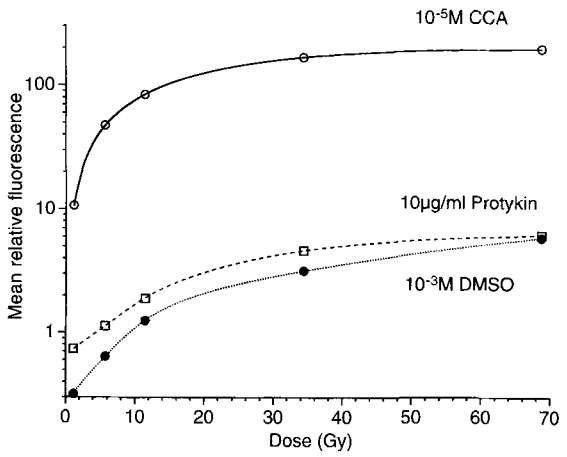

FIGURE 6 Fluorescence of 10^{-5} M aqueous CCA vs. radiation-absorbed dose. The excitation was at 400 nm, the emission at 450 nm, and the slit width was 5/5 nm. The radiation was induced at a rate of 1.14 Gy/min. Relative hydroxyl radical scavenging activity of Protykin and DMSO. CCA (10^{-5} M) was irradiated in the absence (○) and presence of Protykin (10 µ/ml) (□) or DMSO (1 mM) (●). Hydroxyl radical scavenging activity is apparent by the reduction of net mean fluorescence induced by 7-OH•–CCA. Results are (shown as means) representative of three experiments per group. Each experiment was run in triplicate.

Protykin was comparable with that for 25 µg/ml of Trolox.

The induction of 7-OH•–CCA fluorescence by γ-irradiation of CCA is shown in Figure 6. As shown in the figure, with an increasing dose of γ-irradiation, the amount of 7-OH•–CCA fluorescence progressively increased indicating steady formation of OH•. The 7-OH•–CCA fluorescence was effectively blocked by Protykin at a concentration of 10 µg/ml (Figure 6). The scavenging activity of Protykin was compared with that for 1 mM DMSO.

DISCUSSION

The results of this study demonstrated that Protykin-fed rat hearts were more resistant to myocardial ischemic reperfusion injury as compared to those for normal hearts. Protykin group consistently demonstrated better post-ischemic ventricular recovery and reduced myocardial infarction compared to control group. *In vitro* studies using a peroxyl radical generating system by thermal decomposition of AAPH and a OH• radical generating system using γ-irradiation of CCA showed that Protykin is a potent scavenger of both peroxyl and hydroxyl radicals. The free radical scavenging activities of Protykin corroborated with the findings that MDA production was significantly reduced in the hearts of Protykin-fed animals.

Protykin clearly demonstrated cardioprotective properties as evidenced by its ability to improve post-ischemic ventricular function. As expected, the developed pressure and its maximum first derivative as well as aortic flow showed reduced recovery after ischemia/reperfusion compared to the baseline values. Protykin-treated hearts consistently demonstrated improved post-ischemic developed pressure and aortic flow compared to placebo control. A significant amount of infarction was also noticed in the placebo-treated heart which was significantly reduced with protykin. We did not monitor the incidence of arrhythmias in this study. However, studies exist in the literature demonstrating anti-arrhythmic effects of red wine.

Reactive oxygen species have been implicated in the pathophysiology of ischemic heart disease.[3–10] The presence of oxygen free radicals, especially hydroxyl radicals, can be detected directly by HPLC using electrochemical detection technique and by ESR spectroscopy[9] as well as indirectly by the formation of MDA and 8-OH•–DG products.[13,16] The participation of free radicals are also supported from the cardioprotective effects of antioxidants, antioxidant enzymes[5,6,17] and free radical scavengers.[18] Among the reactive oxygen species, superoxide anion (O_2^-) is the most innocent free radical while the hydroxyl radical (OH•) is most cytotoxic to cells. The results of our study demonstrated that Protykin scavenged both hydroxyl and peroxyl radicals which are generated in the ischemic reperfused myocardium.[19,20] In our system, OH• was generated by ionizing radiation.[21]

$OH^{\bullet}$ plays a crucial role in myocardial ischemic reperfusion injury.[20] In spite of their relatively low oxidizing ability compared to $OH^{\bullet}$ radicals, in biological systems, organic peroxyl radicals could be extremely damaging to the tissues.[22] Tissues including myocardium are protected from the detrimental actions of peroxyl radicals by the presence of naturally occurring antioxidants such as bilirubin and biliverdin as well as plasma antioxidants.[23] Ascorbic acid and vitamin E comprise the other potent peroxyl radical traps for the biological systems.[24] Generally, lipid soluble antioxidants can scavenge chain-carrying lipid peroxyl radicals thereby preventing propagation of lipid peroxidation after the initiation of the lipid peroxidation. The results of our study demonstrated that not only was the Protykin a potent scavenger of hydroxyl and peroxyl radicals, but also it reduced the extent of lipid peroxidation in the ischemic reperfused myocardium. These findings seem to be important because these peroxyl radicals are formed *in vivo* in membranes and lipoproteins as intermediate products of lipid peroxidation.

Antioxidant reserve and antioxidant enzymes are significantly reduced after ischemia and reperfusion. For example, reduced amount of SOD, catalase, and glutathione peroxidase enzymes as well as α-tocopherol and ascorbic acid have been found in the ischemic reperfused myocardium.[25,26] The loss of the key antioxidant enzymes and antioxidants thus reduces the overall antioxidant reserve of the heart and make the heart susceptible to ischemia/reperfusion injury. One of the major functions of antioxidants is to block the free radical formation. Thus, the reduced antioxidative defense is likely to be incapable of providing complete protection against increased activities of the reactive oxygen species.

Protykin is a high-potency standardized extract of *trans*-resveratrol and emodin, natural substances found in grapes and other plant foods that have been shown to exhibit a wide range of biological effects. Both resveratrol and emodin have been found to possess antioxidant and antimutagenic properties.[27,28] Resveratrol (3,5,4′-trihydroxystilbene) is a natural phytoalexin found in a wide variety of plant species including grapes. It is abundantly present in the skin of grapes and constitutes one of the major components of red wine.[29] Resveratrol has been found to exert several therapeutic effects which have been attributed to its antioxidant properties. In addition, it inhibits ribonucleotide reductase which may account for its cancer chemopreventive activity.[30] Recently, resveratrol was found to prevent lipid peroxidation and cell death.[31] Resveratrol was also found to stimulate nitric oxide (NO) production in the endothelial cell.[32] In this study, the authors showed both direct and indirect vasodilatory effects on the blood vessels by NO-dependent and NO-independent mechanisms, respectively. Emodin (3-methyl-1,6,8 trihydroxyanthraquinone) is an anthraquinone which exhibits immunosuppressive and vasorelaxant activities.[33] It also possesses antibacterial and antineoplastic properties.[34] Comparison of IC_{50} of emodin with a few other antitumor agents revealed that emodin possesses the strongest inhibitory activity on tumor cell proliferation.[35] In a recent study, emodin was found to inhibit platelet aggregation induced by collagen.[36]

Wine, especially red wine, is rich in polyphenolic compounds which contain several antioxidants including resveratrol and emodin. As mentioned earlier, epidemiological evidence exists to support that the consumption of red wine is associated with reduced incidence of coronary heart disease which is generally known as the *French Paradox*. Proposed mechanisms for cardioprotective effects include an increase in high-density lipoprotein (HDL) cholesterol,[37] reduction/inhibition of platelet aggregation,[38] reduction in clotting factor concentrations,[39] reduction in thromboxane synthesis,[40] increase in vasodilatory prostacyclin synthesis[41] inhibition of low-density lipoprotein (LDL) oxidation[42] and free radical scavenging.[3–10] A Dutch epidemiological study showed that coronary heart disease in elderly males is inversely correlated with

their intake of flavonoids[2] further supporting cardioprotective effects of polyphenolic compounds.[43]

To the best of our knowledge, this is the first report to demonstrate that Protykin consisting of a mixture of resveratrol and emodin possesses cardioprotective properties against ischemic reperfusion injury. Reactive oxygen species including hydroxyl and peroxyl radicals play a crucial role in the pathogenesis of ischemic heart disease. Under normal conditions there is a balance between the formation of pro-oxidants (oxygen free radicals) and the amount of anti-oxidants present. This steady-state condition is interrupted in pathophysiological conditions because of the excessive production of free radicals, or decrease in antioxidants or both. The potent peroxyl and hydroxyl radical scavenging ability makes it a potential therapeutic agent for the amelioration of myocardial injury associated with ischemia and reperfusion.

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