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Does White Wine Qualify for French Paradox? Comparison of the Cardioprotective Effects of Red and White Wines and Their Constituents: Resveratrol, Tyrosol, and Hydroxytyrosol

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It is generally believed that the French paradox is related to the consumption of red wine and not other varieties of wine, including white wine or champagne. Some recent studies have indicated that white wine could also be as cardioprotective as red wine. The present investigation compares the cardioprotective abilities of red wine, white wine, and their principal cardioprotective constituents. Different groups of rats were gavaged with red wine, white wine, resveratrol, tyrosol, and hydroxytyrosol. Red wine and its constituent resveratrol and white wine and its constituents tyrosol and hydroxytyrosol all showed different degrees of cardioprotection as evidenced by their abilities to improve postischemic ventricular performance, reduce myocardial infarct size and cardiomyocyte apoptosis, and reduce peroxide formation. It was discovered in this study that although each of the wines and their components increased the enzymatic activities of the mitochondrial complex (I−IV) and citrate synthase, which play very important roles in oxidative phosphorylation and ATP synthesis, some of the groups were more complex-specific in inducing the activity compared to the other groups. Cardioprotective ability was further confirmed by increased expression of phospho-Akt, Bcl-2, eNOS, iNOS, COX-1, COX-2, Trx-1, Trx-2, and HO-1. The results of this study suggest that white wine can provide cardioprotection similar to red wine if it is rich in tyrosol and hydroxytyrosol.

KEYWORDS: White wine; red wine; resveratrol; tyrosol; hydroxytyrosol; ischemia/reperfusion; peroxide formation; mitochondrial swelling

INTRODUCTION

A perplexing disconnection between the consumption of a high-fat diet and a lower incidence of coronary heart disease among the French people led to the origin of the popular term “French paradox” in 1992, which was explained by the regular consumption of red wine by the French (*1*). It is generally believed that cardioprotection is related to the consumption of red wine, and not other varieties of wine, including white wine, rosé, or champagne. The unique cardioprotective effects have been attributed to several polyphenols, especially to resveratrol, which is present in a relatively high amount in red wine, but not in white wine.

Several recent papers, however, have indicated that some white wines could have cardioprotective abilities similar to those of red wine (*2*). For example, a selected group of white wines from Italy and Germany could reduce oxidative stress and inflammatory response (*3*). Another recent study using a French wine showed a similar degree of protection against early atherosclerosis compared to that produced by sparkling red wine (*4*). A study from our own laboratory also demonstrated that some white wines could reduce myocardial ischemic injury to the same extent as a red wine (*5*). It is becoming increasingly clear that tyrosol and/or hydroxytyrosol, which are present in white wine, may be responsible for cardioprotection (*6*).

Tyrosol and hydroxytyrosol are also present in virgin olive oil (*6*). There are several reports indicating health benefits of tyrosol and hydroxytyrosol (*6*). However, the mechanisms of action of tyrosol compounds against ischemic heart disease are not known. To fill this gap, a study was designed to compare the cardioprotective effects of red versus white wine. For this study, a selected group of red and white wines were used, which were rich in resveratrol and tyrosol, respectively.

Because mitochondrial function, the powerhouse of all cells and energy production, is significantly depressed in the ischemic myocardium, we examined if wines or their components could

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provide cardioprotection by targeting mitochondrial ATP production as well as mitochondrial integrity. In cardiac myocytes, mitochondria play a critical role in both the life and death of the cells. Mitochondrial dysfunction in ischemia/reperfusion (I/R) injury is caused by oxidative stress (*7*). In the postischemic heart, oxygen delivery to the myocardium is not sufficient to meet the need for mitochondrial oxidation during a physiological condition of hypoxia, leaving the mitochondrial electron transport chain (ETC) in a more reduced state. This results in an increase in electron leakage from ETC that in turn reacts with residual molecular oxygen to give superoxide (*8*). Reintroduction of oxygen through reperfusion greatly increases electron leakage along with a decrease in scavenging capacity, leading to superoxide and superoxide-derived oxidants being overproduced in mitochondria (*9*). In addition, a reduction in complex I and complex III activities was shown to result from the elevated level of superoxide (*10*). A recent publication also reported a decrease in the enzymatic activity of complex II and complex III in the postischemic heart (*11*). The molecular changes that take place in mitochondria during I/R are still not clear, but it has been reported that respiratory complexes I, II, III, IV, and V and many Krebs cycle enzymes are all affected by I/R injury (*12*). We, therefore, measured the effects of red and white wines on the activities of the electron transport chain and related enzymes. Finally, to determine the mechanisms of cardioprotection, we measured the signaling components of death versus survival signals.

MATERIALS AND METHODS

Study with Isolated Working Heart Preparation. *Chemicals.* Resveratrol, tyrosol, and hydroxytyrosol were of analytical grade and were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Red [Reunite Lambrusco (Daunia)] and white [Soave Doc Classico 2004 (Le Rive)] wines were received as a presents from Prof. Alberto A. A. Bertelli (University of Milan). All other components were of analytical grade and were obtained from Sigma-Aldrich Chemical Co., 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 NIH 85-23, revised 1996). Sprague−Dawley male rats weighing between 250 and 300 g were fed ad libitum regular rat chow (Harlan Teklad, Madison, WI) with free access to water until the start of the experimental procedure. All of the compounds were given by gavage for 14 days and on th 15th day, the animals were sacrificed for isolated working heart preparation. The rats were randomly assigned to one of the following groups: (i) control; (ii) ischemia [30 min]/reperfusion [2 h] [I/R]; (iii) I/R + ethanol (1 mL, 12%); (iv) I/R + white wine [6.5 mL/kg]; (v) I/R + red wine [6.5 mL/kg]; (vi) I/R + resveratrol [2.5 mg/kg]; (vii) I/R + tyrosol [2.5 mg/kg]; (viii) I/R + hydroxytyrosol [2.5 mg/kg]. The resveratrol, tyrosol, and hydroxytyrosol were dissolved in 12% ethanol. The concentrations of the active compound of red and white wine are shown in **Table 1**. In these experiments 120 animals were used; during treatment 2 animals died. In **Figure 1A** is shown the treatment protocol; panel **B** presents the experimental protocol.

Isolated Working Heart Preparation. After 14 days of treatment, the animals were sacrificed. All of them were anesthetized with sodium pentobarbital (80 mg/kg, ip) (Abbott Laboratories, North Chicago, IL), and heparin sodium (500 IU/kg, iv) (Elkins-Sinn Inc., Cherry Hill, NJ) was used as an anticoagulant. After a sufficient depth of anesthesia had been confirmed, a thoracotomy was performed, and hearts were removed, cannulated through the aorta, and perfused with the KHB buffer in the retrograde Langendorff mode at 37 °C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period as described previously (*13*). The perfusion medium consisted of a modified Krebs−Henseleit bicarbonate buffer (millimolar concentration: sodium chloride, 118; potassium chloride, 4.7; calcium chloride, 1.7; sodium bicarbonate, 25; potassium dihydrogenphosphate, 0.36; magnesium sulfate, 1.2; and glucose, 10), and after its oxygenization that pH was 7.4 at 37 °C. Following the washout period, the Langendorff preparation was switched to the working mode for 10 min with a left atrial filling pressure of 17 cm H_2O and aortic afterload pressure of 100 cm H_2O as previously described. At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The hearts were then subjected to 30 min of global ischemia followed by 2 h of reperfusion in the antegrade working mode (*13*). The ischemia was initiated by clamping the left atrial inflow and aortic outflow lines at points close to their origins, and reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines.

Table 1. Constituents of Red [Reunite Lambrusco (Daunia)] and White [Soave Doc Classico 2004 (Le Rive)] Wines

constituent	white wine	red wine
shikimic acid	19.0 mg/L	
hydoxytyrosol	2.69 mg/kg	
tyrosol	17.06 mg/kg	nq[a]
vanillic acid	0.99 mg/kg	
caffeic acid	7.15 mg/kg	10.5 mg/L
ferulic acid	1.42 mg/kg	nq
p-coumaric acid	0.72 mg/kg	3.4 mg/L
quercetin	<0.1 mg/kg	2.1 mg/L
resveratrol		2.81 mg/L
gallic acid		10.1 mg/L
myricetin		2.1 mg/L

[a] nq, not quantified.

Cardiac Function Assessment. Baseline cardiac functional data were recorded after 10 min of working mode perfusion. After 60 and 120 min of reperfusion, the functional data were recorded to monitor the recovery of the left ventricular parameters. Aortic flow (AF) was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL), and coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart. Aortic pressure was monitored during the whole experiment using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH) connected to a sidearm of the aortic cannula; the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA) (*13*). Heart rate (HR), left ventricular developed pressure (LVDP), and the first derivative of developed pressure (dp/dt) were all calculated from the continuously obtained pressure signal.

Infarct Size Estimation. At the end of the 2 h reperfusion, 35 mL of a 1% (w/v) solution of triphenyl tetrazolium chloride (TTC) in phosphate buffer was infused through the aortic cannula for 20 min at constant pressure at 37 °C and stored at −70 °C for subsequent analysis (*13*). Sections (0.8 mm) of frozen heart were fixed in 2% paraformaldehyde, placed between two coverslips, and digitally imaged using a Microtek ScanMaker 600z. To quantitate the areas of infarct in pixels, NIH image 5.1 (a public-domain software package) was used. The infarct size was quantified and expressed in pixels.

TUNEL Assay for Assessment of Apoptotic Cell Death. Immunohistochemical detection of apoptotic cells was carried out using the TUNEL method (*13*) (Promega, Madison, WI). The heart tissues were immediately put in 10% formalin and fixed in an automatic tissue fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic molds, and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed, and tissues became embedded in paraffin on the plastic molds. Prior to analysis of tissues for apoptosis, tissue sections were deparaffinized with xylene, washed, and rehydrated by sequential wash with different concentrations of ethanol (absolute, 95%, 85%, 70%, 50%). Then the TUNEL staining was performed according to the manufacturer's instructions. The fluorescence staining was viewed with a fluorescence microscope (Axioplan2 Imaging) (Carl Zeiss Microimaging Inc., New York) at 520 ± 20 nm for green fluorescence of

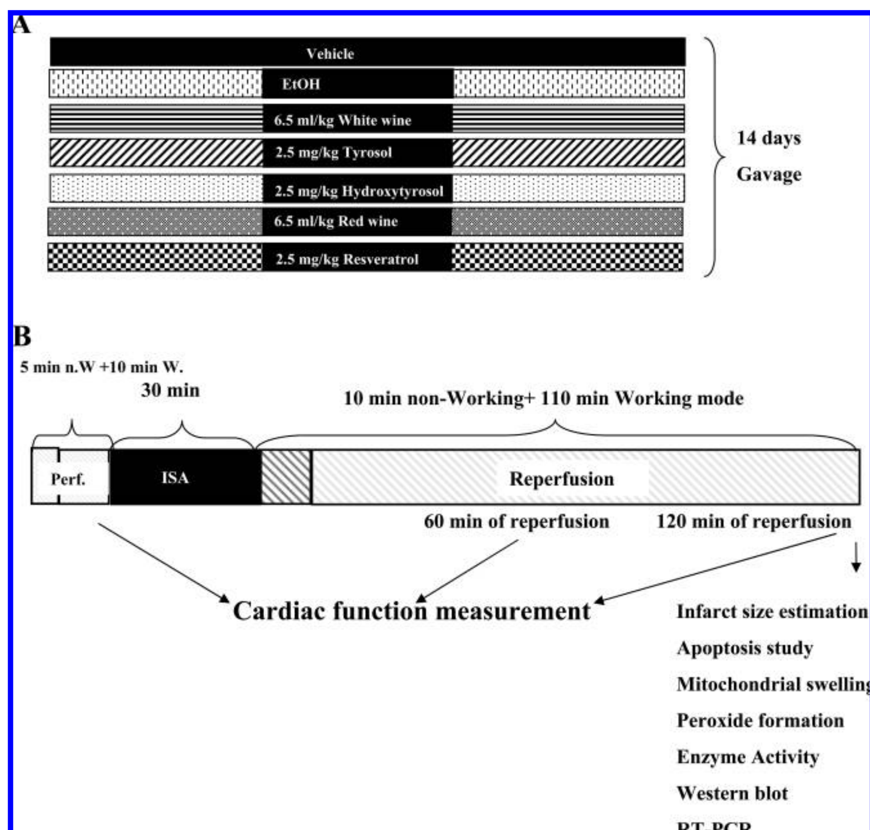

Figure 1. Schematic representation of treatment protocol (**A**) and perfusion protocol of different groups of heart (**B**). Perf, perfusion; ISA, ischemia.

fluorescein and at 620 nm for red fluorescence of propidium iodide. The number of apoptotic cells was counted throughout the slides and expressed as a percent of total myocyte population.

Western Blot Analysis. Left ventricles from the hearts were homogenized in 1 mL of buffer A (25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail) (*13*). The homogenates were centrifuged at 2000 rpm at 4 °C for 10 min. The supernatant was centrifuged at 10000 rpm at 4 °C for 20 min. The resultant supernatant was the cytosolic fraction. The cytosolic extracts were aliquoted, snap frozen, and stored at −80 °C until use. Total protein concentrations in cytosolic extracts were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

The cytosolic proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk and probed with primary antibody 1:1000 dilution overnight. The following primary antibodies were obtained from Cell Signaling Technology (Boston, MA): Bcl-2, cytochrome *c*, and Bcl2-associated X protein (Bax). The primary antibodies Phospho-Akt, Akt, inducible nitric oxide synthase (i-NOS), endothelial nitric oxide synthase (e-NOS), heme oxygenase-1 (HO-1), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The protein bands were detected using horseradish peroxidase conjugated secondary antibody (1:2000 dilution) and Western Blot luminol reagent (Santa Cruz Biotechnology). GAPDH was used for the cytosolic loading control. The bands were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against the loading control.

Total RNA Isolation and RT-PCR. Total RNA was isolated from the left ventricle with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and dissolved in 20 μL of DEPC-treated water. Total RNA concentration was determined by measuring the optical density at 260 nm. Reverse transcription and Polymerase Chain Reaction (RT-PCR) were performed with RETRO-script (Ambion, Austin, TX), according to the manufacturer's instructions. Two micrograms of RNA was used to prepare cDNA. The following primers were used in our study: thioredoxin-1, forward 5-′GCCAAAATGGTGAAGCTGA-3′, reverse 5′-CTGGCAGTCATC-CACGTCT-3′; thioredxin-2, forward 5′-GCATCCTGAGCACCTC-CTAC-3′, reverse 5-′GAGCCACATGTGTGTGTGTG-3′; GAPDH, forward 5′-AGACAGCCGCATCTTCTTGT-3′, reverse 5′-CTTGCCG-TGGGTAGAGTCAT-3′.

PCR reactions were performed in 25 μL reaction volume with the programs 95 °C for 4 min (denaturation), 94 °C for 30 s, AT 55 °C for 30 s, 72 °C for 1 min (30 cycles), and 72 °C for 5 min. The PCR products were visualized on a UV-transilluminator and digitalized after electrophoresis on 2% agarose gel containing ethidium bromide.

Study with Living Mitochondria. *Isolation of Living Mitochondria.* At the end of each experiment, the hearts were subjected to the protocol described by Das et al. (*14*). In short, the hearts were placed into ice-cold sucrose buffer containing 300 mM sucrose, 10 mM Tris-HCl, and 2 mM EGTA at pH 7.4. Each heart was homogenized in 3.5 mL of sucrose buffer using a Polytron homogenizer (Brinkmann Homogenizer, Switzerland). The homogenates from three hearts were diluted to 40 mL with sucrose buffer containing 5 mg/mL of bovine serum albumin (BSA) and centrifuged at 4 °C for 2 min at 2000*g* to sediment cell debris. The supernatant was extracted and centrifuged for 5 min at 10000*g* (4 °C) to settle a crude mitochondrial pellet. The pellet was resuspended in 12 mL of ice-cold 19% (w/v) Percoll (Sigma-Aldrich) gradient and sucrose buffer and centrifuged at 14000*g* for 10 min (4 °C), forming a very loose pellet. The supernatant was carefully drawn off and discarded. The loose pellet was resuspended in 40 mL of sucrose buffer and centrifuged at 10000*g* for 5 min (4 °C). The final mitochondria pellet was resuspended to about 30 mg of protei,/ mL and kept on ice until use (<3 h).

Mitochondria Swelling Assay. The opening of the mitochondrial permeability transition pores (MPTP) was analyzed by mitochondrial swelling. The changes in mitochondria volume were monitored spectrophotometrically at 25 °C as a decrease in light absorbance at 520 nm with time (*14*). The 520 nm wavelength is an isosbestic point for the mitochondrial cytochromes and is not influenced by changes in the redox state. The mitochondria swelling assay contained 125 mM KCl, 20 mM MOPS, 10 mM Tris, 0.5 mM EGTA, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 5 mM succinate, 1 μM oligomycin, 0.2 μM rotenone, 1 mM ATP, and 10 mM $KHCO_3$ (pH 7.2), in the reaction cuvette. The

addition of 100 μM 5-hydroxydecanoic acid (5-HD) and 50 μM valinomycin (Val) to the assay in the reference cuevette was used to monitor the blocking and opening of the mitochondrial K_{ATP} channels. The addition of 1 mg of mitochondrial protein to the 1 mL assay initiated the reaction.

Determination of Peroxide Formation. The PeroXOquant Quantitative Peroxide Assay kit (Pierce, Rockford, IL) was used to detect the peroxide production from the oxidation of ferrous to ferric ion in the presence of xylenol orange; 0.1 mg of mitochondrial protein (diluted to 20 μL with distilled water) was added to 200 μL of the reagent mixture. The sample was incubated for 30 min at room temperature. The absorbance was taken at 595 nm using an MR 600 microplate reader (Dynatech, Chantilly, VA). A standard curve ranging from 0 to 1000 μM peroxide was constructed prior to each experiment. The amount of peroxide formation in experimental samples was calculated from a standard curve prepared with peroxide and expressed as moles per milligram of protein.

Enzyme Activities. Enzyme activities for citrate synthase and complexes I–IV were performed using 1 mL assays at room temperature in a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA).

Citrate Synthase Assay. The citrate synthase activity was measured by monitoring an increase of absorbance at 412 nm at 25 °C (*15*). The 1 mL reaction assay contained 100 mM Tris (pH 8.0), 100 μM acetyl CoA, 10 μM DTNB, and 50 μg of mitochondrial protein. The reaction was initiated by the addition of 100 μM potassium oxalate. The activity was calculated using 13600 M^{-1} cm^{-1}. The specific activity of the enzyme is expressed as micromolar citrate produced per minute per milligram of mitochondrial protein.

Complex I (NADH Dehydrogenase) Assay. The activity of complex I was measured by monitoring an increase of absorbance at 340 nm at 25 °C (*15*). The 1 mL reaction assay contained 50 mM phosphate buffer (pH 7.4), 0.17 mM NADH, 0.6 mM ferricyanide, and 0.1% Triton X-100. The reaction was initiated by the addition of 30 μg of mitochondrial protein. The activity was calculated using 6220 M^{-1} cm^{-1}. The specific activity of the enzyme is expressed as micromolar reduced NADH per minute per milligram of mitochondrial protein.

Complex II (Succinate–Cytochrome c Reductase) Assay. The malonate-sensitive succinate–cytochrome *c* reductase activity of complex II was measured by monitoring the absorbance increase of the reduction of cytochrome *c* at 550 nm at 25 °C (*16*). The 1 mL reaction assay contained 0.1 M Tris (pH 7.4), 1.6 mg of ferricytochrome *c*, 1 mM sodium cyanide, and 20 mM sodium succinate. The reference cuvette contained the reaction assay plus 27 mM sodium malonate. The reaction was initiated by adding 150 μg of mitochondrial protein. The activity was calculated using 19.1 mM^{-1} cm^{-1}. The specific activity of the enzyme is expressed as nanomoles of cytochrome *c* reduced per minute per milligram of mitochondrial protein.

Complex III (Ubiquinol–Cytochrome c Reductase) Assay. The activity of complex III was measured by monitoring the absorbance increase of the reduction of cytochrome *c* at 550 nm at 25 °C. One milligram of mitochondrial protein was incubated in 1 mL of 50 mM PBS buffer (pH 7.2). Three micrograms of preincubated protein was added to 1 mL of reaction assay containing 3 mM sodium azide, 1.5 μM rotenone, 50 μM ferricytochrome *c*, and 50 mM phosphate buffer (pH 7.2). The reaction was initiated by adding 30 μM decylubiquinol. The activity was calculated using 19.1 mM^{-1} cm^{-1}. The specific activity of the enzyme is expressed as nanomoles of cytochrome *c* reduced per minute per milligram of mitochondrial protein (*17*).

To prepare decylubiquinol for complex III activity measurement, 2 mg of decylubiquinone was dissolved in 1 mL of diethyl ether and then mixed with 2 mL of 2.3 mM dithionite in 1 M potassium phosphate buffer (pH 7.0) (*18*). The aqueous and organic phases were allowed to separate. The bottom aqueous layer was discarded, and the process was repeated. The colorless organic layer was washed twice with 10 mM HCl solution saturated with NaCl and passed through a column of anhydrous sodium sulfate. The solution was evaporated, and the decylubiquinol powder was redissolved in 1 mL of ethanol solution containing 6 mM HCl.

Complex IV (Cytochrome c Oxidase) Assay. The activity of complex IV was measured by monitoring the oxidation of cytochrome *c* at 550 nm with 580 nm as the reference wavelength at 25 °C (*19*). About 1 mg of mitochondrial protein was incubated for 5 min on ice in 1 mL of buffer containing PBS (100 mM, pH 7.2), 25 mM NaCl, and 15 g/L dodecylmaltoside; 2.5 μg of preincubated protein was added to 1 mL of reaction assay containing PBS (50 mM, pH 7.2) and 1 mM dodecylmaltoside. The reaction was initiated by adding 15 μM ferrocytochrome *c*. The activity was calculated using 19.1 mM^{-1} cm^{-1}. The specific activity of the enzyme is expressed as nanomoles of cytochrome *c* oxidized per minute per milligram of mitochondrial protein.

Statistical Analysis. The values for myocardial functional parameters, total and infarct volumes, and infarct sizes as well as cardiomyocyte apoptosis are all expressed as the mean ± standard error of mean (SEM). Analysis of variance test followed by Bonferroni's correction was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of the treated groups were compared with those of the control group by a modified *t* test. The results were considered to be significant if $p < 0.05$.

RESULTS

Cardioprotection with Red Wine, White Wine, and Their Constituents. The results of our study using isolated working heart preparation documented that red wine and its constituent resveratrol as well as white wine and its constituents tyrosol and hydroxytyrosol provided significant amounts of cardioprotection as evidenced by their abilities for postischemic ventricular recovery and reduction of cardiac infarct size and cardiomyocyte apoptosis. As shown in **Figure 2**, the cardiac function, except for heart rate and coronary flow, was reduced during the reperfusion as expected. White wine, red wine, tyrosol, hydroxytyrosol, and resveratrol improved postischemic ventricular function significantly ($p < 0.05$, 95% CI). There were no differences between the groups.

Figure 3 shows the amount of myocardial infarct produced at the end of reperfusion with or without treatment. Thirty minutes of ischemia followed by 120 min of reperfusion produced about 35% infarct in the control-IR sample. Infarct sizes were significantly ($p < 0.05$, 95% CI) lower for all of the treatment groups compared to control-IR. Hydroxytyrosol and resveratrol appeared to be most effective in reducing the infarct size.

Because myocardial infarct is contributed from both necrosis and apoptosis, we also measured cardiomyocyte apoptosis as described under Materials and Methods. As depicted in **Figure 4A**, the number of apoptotic cardiomyocytes increased significantly ($p < 0.05$, 95% CI) in the control-ischemic reperfused myocardium. All of the treatments were effective in the reduction of apoptotic cell death. However, similar to the myocardial infarct size, hydroxytyrosol and resveratrol were the most effective for the reduction of cardiomyocyte apoptosis.

Because the most plausible mechanism of cardiomyocyte apoptosis involves release of cytochrome *c* from the mitochondria followed by the activation of caspases, we determined the cytochrome *c* in the cytosol by Western blot analysis. As shown in **Figure 4B**, a significant ($p < 0.05$, 95% CI) amount of cytochrome *c* was found in the cytosolic fractions of the control-ischemic reperfused myocardium. All of the treatment modalities reduced the amount of cytochrome *c* that was released into the cytosol, significantly.

Mitochondrial Swelling Assay. The opening of mitochondrial permeability transition pore [MPTP] changes the mitochondrial volume as mitochondria swells. Swelling of mitochondria ruptures the outer mitochondrial membrane and releases apoptosis-inducing factors leading to cell death. As shown in **Figure 5**, ischemia/reperfusion significantly increased the swelling of mitochondria, and such swelling was significantly

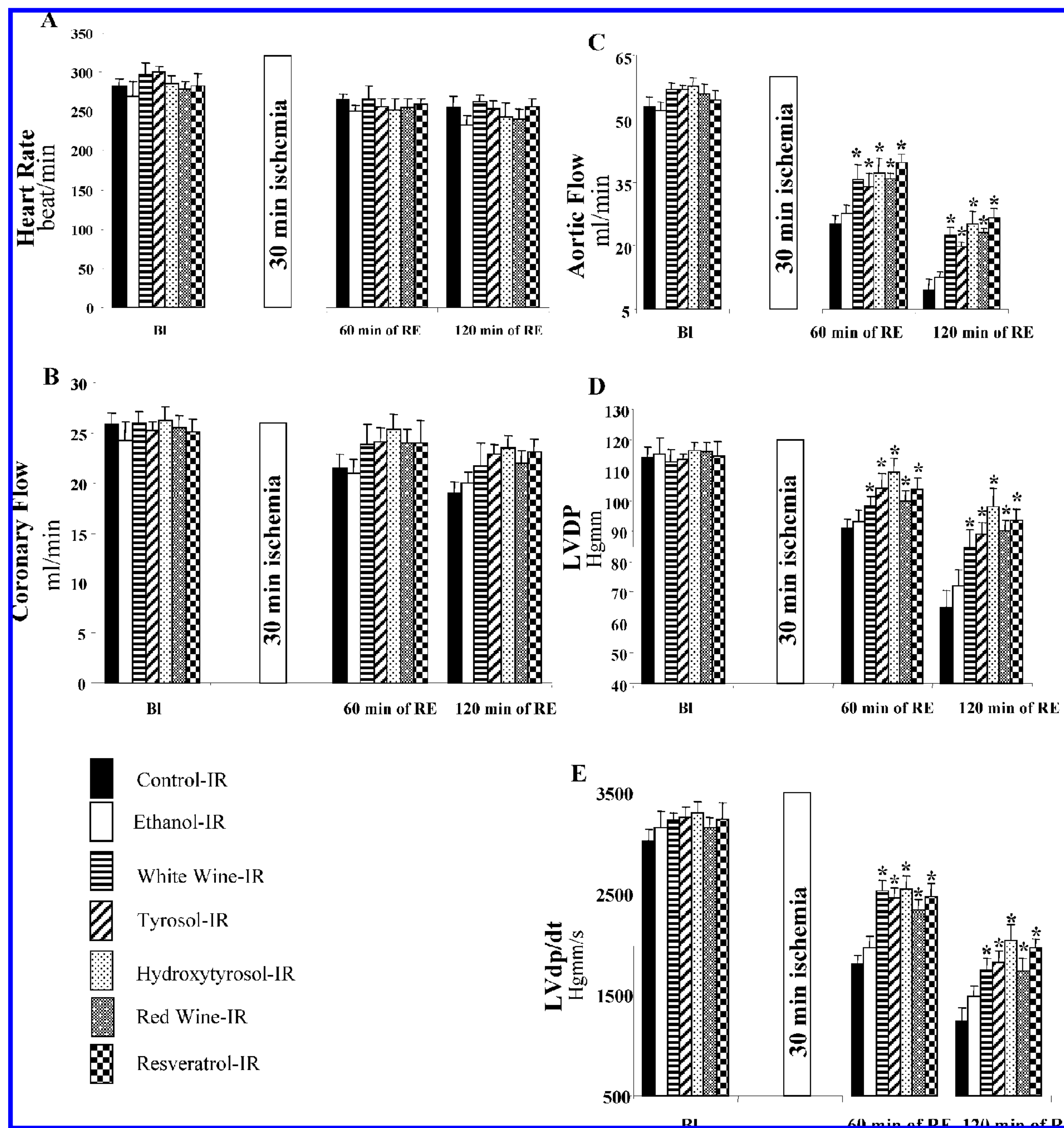

Figure 2. Effect of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on the postischemic ventricular performance. Heart rate (**A**), coronary flow (**B**), aortic flow (**C**), left ventricular developed pressure (**D**), and first derivative of left ventricular developed pressure (**E**) were monitored. $n = 6$ in each group. The results shown are mean ± SEM. Comparisons were made to the values of drug-free control group. ∗, $p < 0.05$ vs I/R. Bl, baseline.

($p < 0.05$, 95% CI) reduced with wines and their constituents. Red wine and resveratrol reduced mitochondria swelling 6- and 3-fold, respectively. Similarly, white wine, tyrosol, and hydroxyltyrosol reduced mitochondria swelling by 4-, 6-, and 4-fold, respectively.

Measurement of Peroxide Formation. As expected, peroxide formation was significantly ($p < 0.05$, 95% CI) increased in the control-I/R heart (**Figure 6**), but wines and their constituents reduced the peroxide formation. Red wine and resveratrol reduced peroxide formation by 7- and 9-fold, respectively. White wine, tyrosol, and hydroxytyrosol reduced peroxide formation by 7-, 6-, and 9-fold, respectively.

Mitochondrial Complex (I−IV) Activity Assay. Mitochondrial complexes play a very important role in the function of ETC as well as ATP synthesis and oxidative phosphorylation. The results showed that control-I/R inhibited ($p < 0.05$, 95% CI) enzymatic activity of complexes I−IV as expected and that wines and their constituents restored the activity (**Figure 7A−D**). In the case of complex II activity, tyrosol was the most potent in restoring complex II enzymatic activity, whereas white wine was least potent. Red wine and resveratrol increased the activity by 180 and 205%, respectively, compared to control-I/R, whereas white wine, tyrosol, and hydroxytyrosol increased the activity by 140, 215, and 190% activity, respectively. Results also showed that white wine was the most potent in restoring complex III and complex IV enzymatic activities. In the case of complex III, red wine and resveratrol showed 190 and 194% increases in activity, respectively, compared to control-I/R, whereas white wine, tyrosol, and hydroxytyrosol showed 200, 142, and 148% increases in activity, respectively. For complex IV, white wine, tyrosol, and hydroxytyrosol showed increases of 23-, 21-, and 11-fold in activity, respectively, whereas red

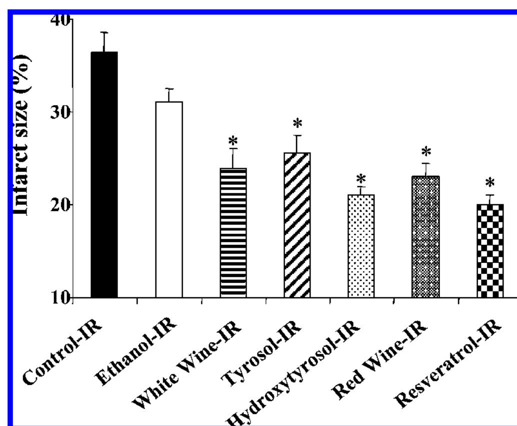

Figure 3. Effect of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on the infarct size determined by triphenyl tetrazolium chloride (TTC) method. $n = 6$ in each group. The results shown are mean ± SEM. Comparisons were made to the values of drug-free control group. ∗, $p < 0.05$ vs I/R.

wine and reveratrol showed, respectively, 7- and 10-fold increases in activity. For complex I, the results were expressed as the formation of reduced NADH. Control-I/R showed a high level of reduced NADH, indicating that the activity was low. Hydroxytyrosol showed the most potent action in restoring complex I activity followed by tyrosol, white wine, red wine, and resveratrol.

Measurement of Citrate Synthase Activity. Citrate synthase acts as a pacemaking enzyme in the first step of citric acid cycle. Citrate synthase activity was significantly ($p < 0.05$, 95% CI) reduced in the control-I/R heart, as expected (**Figure 7E**). The wines and their constituents restored the citrate synthase activity. Red wine was the most potent in restoring citrate synthase activity. Red wine and resveratrol showed 3- and 2-fold increases, respectively.

Estimation of Akt, Phospho-Akt, Bax, and Bcl-2. Because the activation of Akt comprises the major signaling pathway leading to cell survival, we determined the effects of the treatments on Akt activation. As shown in **Figure 8A**, the ratio of phospho-Akt/Akt was reduced significantly for the control-I/R group compared to control-BL. Both red and white wines as well as their constituents could induce activation significantly ($p < 0.05$, 95% CI). The maximum Akt phosphorylation was noted for red wine and resveratrol. Similar to Akt activation, an increase in the ratio of Bcl-2/Bax indicates cell survival. As shown in **Figure 8A**, the Bcl-2/Bax ratio was significantly depressed in the hearts subjected to control-I/R. The ratio was significantly increased with wines and their constituents, most notably for red wine, resveratrol, and hydroxytyrosol, which were consistent with the results of Akt activation.

Estimation of eNOS, iNOS, HO-1, COX-1, and COX-2. Because red wine and resveratrol were previously found to activate both eNOS and iNOS as well as phase 2 enzymes including HO-1, COX-1, and COX-2, we compared the activities of these enzymes against various treatments. As shown in **Figure 8B**, HO-1 activity was negligible after ischemia/reperfusion, which was increased in the treated groups. White wine and tyrosol minimally, but significantly ($p < 0.05$, 95% CI), increased HO-1 activity, whereas hydroxytyrosol, red wine, and resveratrol enhanced HO-1 to a greater extent. The most significant increase ($p < 0.05$, 95% CI) was found with resveratrol, which also significantly induced the expression of both iNOS and eNOS. Induction of iNOS was noted only for hydroxytyrosol, red wine, and resveratrol, whereas the increased expression of eNOS was found for all treatments, the maximal enhancement being again for red wine and resveratrol. Interestingly enough, the maximal COX-1 expression was noted for white wine, red wine, and hydroxytyrosol, whereas COX-2 expression was high in tyrosol, hydroxytyrosol, and red wine. Resveratrol and white wine showed less expression for COX-2.

Estimation of mRNA Transcripts of Trx-1 and Trx-2. Because it is known that resveratrol can provide cardioprotection by inducing the thioredoxin gene, we compared the mRNA expression level of Trx-1 and Trx-2 by RT-PCR. As shown in **Figure 8C**, ischemia/reperfusion reduced the transcripts of mRNAs of both Trx-1 and Trx-2, but white wine, tyrosol, hydroxytyrosol, red wine, and resvaratrol treatment can prevent this reduction of Trx gene expression. In the case of Trx-2 gene expression, resveratrol, red wine, hydroxytyrosol, and tyrosol not only prevent the reduction of this gene expression but also up-regulate Trx-2 over the control values ($p < 0.05$, 95% CI).

DISCUSSION

The present study was based on the results of several recent papers that had indicated that some white wines could have the same health benefits as red wines. Much of the cardioprotective effects of red wine are derived from the resveratrol, which is present in red wines, but not in white wine, whereas the health benefits of white wines are presumably derived from the presence of tyrosol and hydroxytyrosol (*3*). The white and red wines used in this study were carefully selected to ensure the presence of sufficient amounts of resveratrol in red wine and tyrosol and hydroxytyrosol in white wine. There are several salient features in this study: (i) the results clearly show that the red wine and white wine used in this study are comparably cardioprotective in the animal model; (ii) comparison of the cardioprotective effects of the wines with their ingredients revealed that resveratrol, tyrosol, and hydroxytyrosol possess degrees of cardioprotective abilities similar to those of the wines; (iii) from postischemic ventricular recovery, myocardial infarct size, and cardiomyocyte apoptosis, hydroxytyrosol appears to be the best followed by resveratrol, tyrosol, red wine, and white wine; (iv) studies with living mitochondria documented that for the reduction of myocardial swelling, hydroxytyrosol was the best, followed by resveratrol and red wine and then tyrosol and white wine; (v) no difference has been found in their (wines and their components) ability to reduce peroxide formation; (vi) as expected, the activities of all the mitochondrial complexes including complexes I−IV were drastically reduced after the hearts had been subjected to ischemia/reperfusion; both red wine and white wine as well as resveratrol, tyrosol, and hydroxytyrosol could improve the activities of the mitochondrial complexes.

A significant number of papers are available in the literature indicating the improvement of cardiovascular health with regular consumption of a moderate (therapeutic) amount of red wine (*1*). A growing body of evidence also supports the notion that not only does red wine improve cardioprotection, it also exerts anti-inflammatory response, slows memory loss, protects brain damage after a stroke (*20*), and extends human life expectancy (*21*). The cardioprotective role of red wine was first published in 1991 and resulted in the origin of the so-called "French paradox". The epidemiologic studies were subsequently supported by laboratory investigations, which demonstrated cardioprotective properties of red wine and its components (*1*). Proposed mechanisms for such cardioprotective effects of red wine consumption include increase in high-density lipoprotein (HDL) cholesterol (*22*), reduction/inhibition of platelet aggrega-

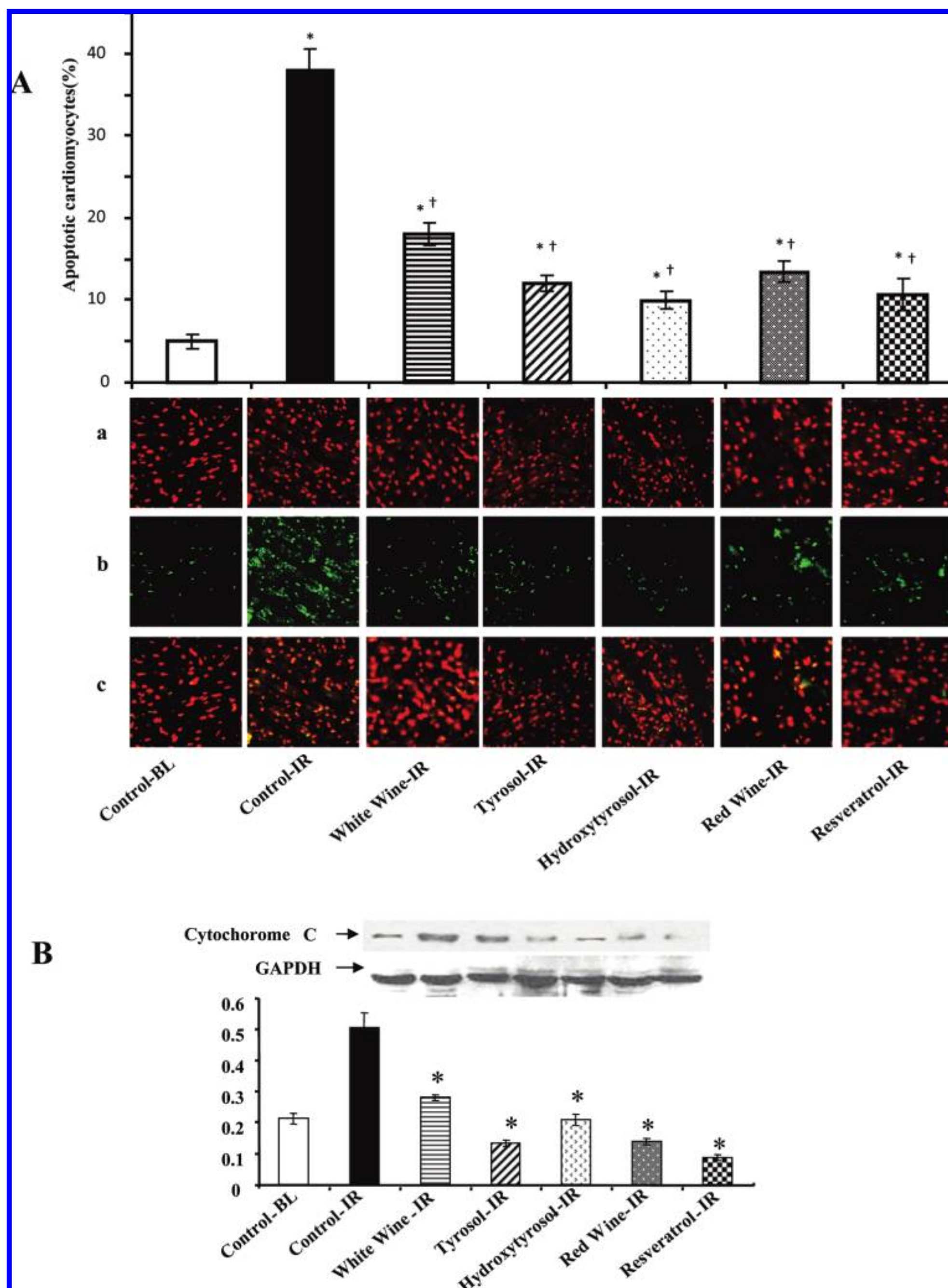

Figure 4. (**A**) Effects of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on cardiomyocyte apoptosis. $n = 6$ for each group. Panel **a** is total number of cells (red channel). Panel **b** is total number of apoptotic cells (green channel). Panel **c** is merged imaged of panels **a** and **b** (yellow dots in merged channel are the apoptotic cells). Values are mean ± SEM. ∗, $p < 0.05$ vs control. †, $p < 0.05$ vs I/R. Representative photomicrographs are shown below the bar graphs. (**B**)Western blot analysis of cytosolic levels of cytochrome *c* in heart tissue obtained from control-BL, control-I/R, and white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol. Values are mean ± SEM. ∗,$p < 0.05$ vs I/R. Representative photomicrographs are shown above the bar graphs.

tion (*23*), reduction in clotting factor concentrations (*24*), reduction in vasoconstrictatory thromboxane synthesis, increase in vasodilatory prostacyclin synthesis (*25*), inhibition of low-density lipoprotein (LDL) oxidation (*26*), and free radical scavenging (*27*), among others. A sufficient body of evidence now supports that moderate wine consumption (one to two glasses a day) is capable of imparting protection against atherosclerosis and myocardial infarction. The results of our study support this concept as we used equivalent amounts of wine in our study.

Wines contain a number of polyphenolic antioxidants including phenolic acids (*p*-coumaric, cinnamics, caffeic, gentistic, ferulic, and vanillic acids), proanthocyanidins, catechin, and epicatechin quercetin. Red wines are also rich in resveratrol (3,5,4-trihydroxystilbene), which is generally present in the amount of 1−10 mg/L depending on the variety of red wine. In most varieties of U.S. red wine, the concentration of resveratrol rarely exceeds 1 mg/L (*28*). Italian red wines contain much higher amounts of resveratrol, ranging from 5 to 10 mg/L (*29*). Among the components of red wine, resveratrol has received much attention as supplying most of the cardioprotective property of red wine. For example, although resveratrol is a poor antioxidant in vitro, it functions as a potent antioxidant in vivo (*20*). Similar to red wine, resveratrol can increase HDL,

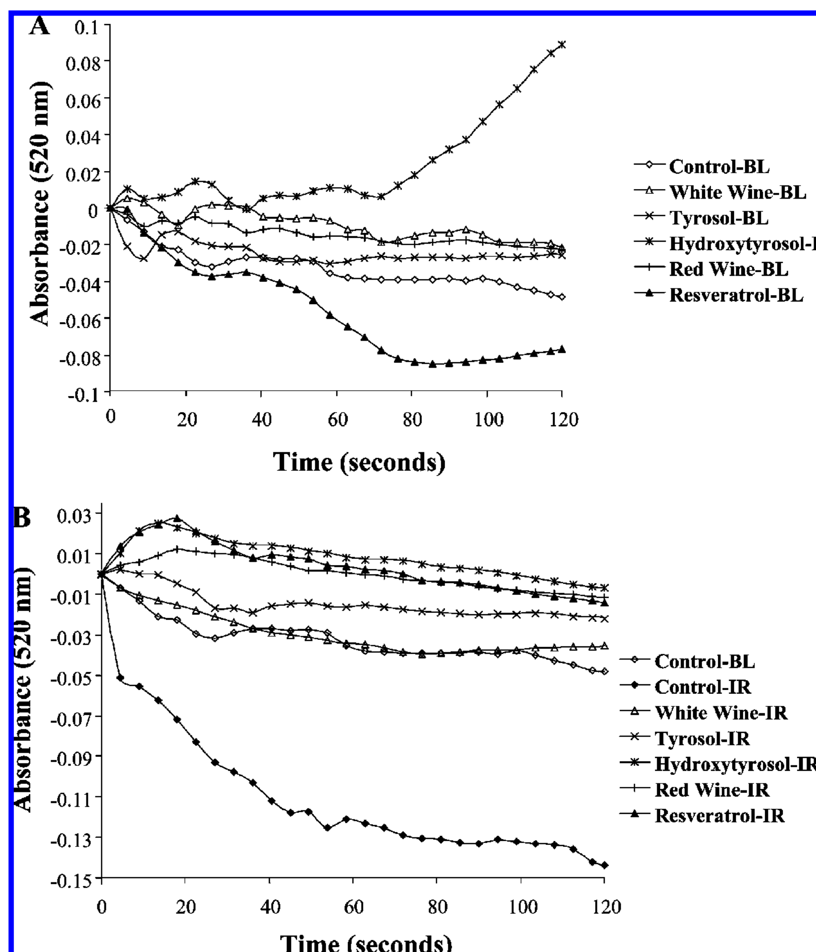

Figure 5. Effect of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on mitochondria swelling. Panels **A** and **B** compare the swelling of the mitochondria matrix volume for treated heart samples at baseline (BL) and induced by ischemic/reperfusion (IR), respectively. Panels **A** and **B** represent data collected from at least six separate experiments with different mitochondrial preparations.

lower LDL, exert anti-inflammatory action, and function as a potent vasodilator through its ability to stimulate NO synthesis (*30, 31*).

Evidence is rapidly emerging supporting the cardioprotective role of white wine. Initial studies focused on cardioprotection with red wines only because white wines contain very little to no resveratrol. The reason for this is that red wines are produced from the grapes that include their skins, which contain resveratrol, whereas white wines are made from grapes without their skins. Nevertheless, one of our earlier studies performed by feeding white wine to rats demonstrated cardioprotective abilities of certain varieties of white wines (*5*). Consequently, several other studies also found cardioprotective abilities of white wines (*2*). More recently, our study provided direct evidence that grapes without skin could possess cardioprotective properties, and hence the cardioprotective abilities could be translated into white wines (*32*).

Although white wines do not contain resveratrol to any appreciable amount, they contain other cardioprotective antioxidants such as cinnamics acid, tyrosol, and hydroxytyrosol. In this study, almost all varieties of Italian wines were found to contain hydroxytyrosol. Tyrosol and hydroxytyrosol are phenolic compounds that are also found in olive oil, which are present in olives either as free or conjugated forms as steroids or aglycones (*6*). Tyrosol and hydroxytyrosol possess several cardioprotective properties including their abilities to protect LDL against oxidation (*33*), inhibit platelet aggregation (*34*), prevent formation of pro-inflammatory agents by activated leukocytes (*3*), and inhibit endothelial expression of tissue factor (*35*). Although alcohol could also provide cardioprotection by reducing LDL and increasing HDL as well as by exerting preconditioning-like effect, it appears to be unlikely that the small amount of alcohol present in our samples would exert any significant cardioprotection as our results indicated no improvement in cardioprotection with control alcohol over the baseline control.

In the present investigation, peroxide formation was increased in control-I/R heart, but wines and their constituent products decreased I/R-mediated peroxide formation in the heart. Resveratrol was the most potent compound in decreasing the peroxide level followed by hydroxytyrosol, red wine, white wine, and tyrosol. According to the earlier papers, it was also found that I/R inhibited the enzymatic activity of complexes II, III, and IV and wine and their constituents restored the activity. In the case of complex II activity, tyrosol was the most potent in restoring complex II enzymatic activity followed by resveratrol, hydroxytyrosol, and red wine, whereas white wine was the least potent in restoring complex II activity. For complex III activity, white wine was most potent in restoring complex III enzymatic activity followed by resveratrol, red wine, hydroxytyrosol, and tyrosol. For complex IV activity, white wine was most potent in restoring complex IV enzymatic activity followed by tyrosol, hydroxytyrosol, resveratrol, and red wine.

From these results it can be concluded that both red and white wines and their constituents protect the heart by decreasing peroxide formation and restoring the enzymatic activity of mitochondrial ETC complexes, which helps to maintain cellular

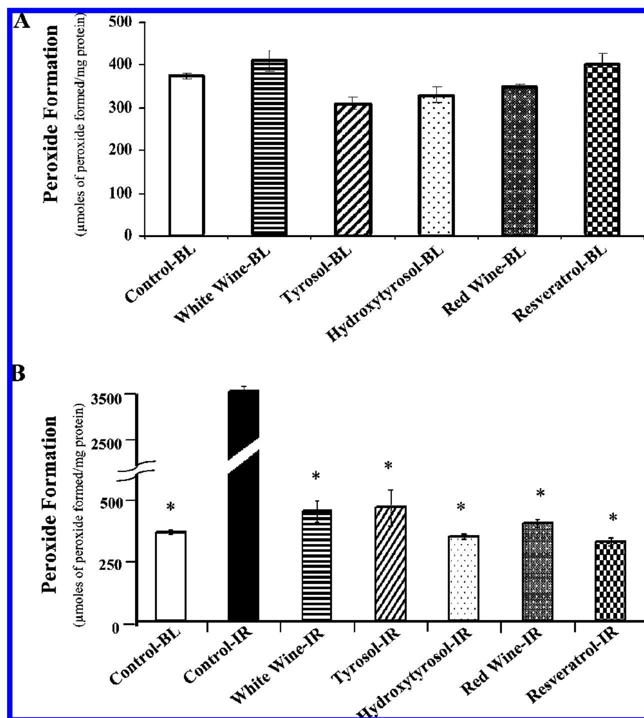

Figure 6. Effect of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on peroxide formation. Panels **A** and **B** compare peroxide formation for treated heart samples at baseline (BL) and induced by ischemic/reperfusion (IR), respectively. The panels represent data collected from at least eight separate experiments with different mitochondrial preparations. Error bar represents standard error about the mean ± SEM. ∗, $p < 0.05$ vs I/R.

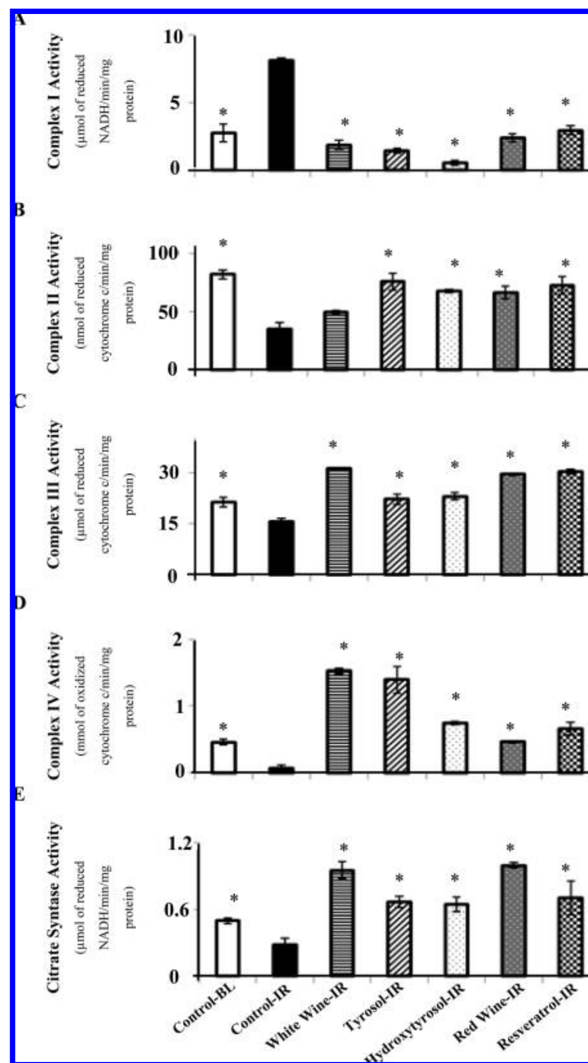

Figure 7. Effect of white wine, tyrosol, hydroxytyrosol, red wine, and resveratrol on the mitochondrial complexes (I−IV) and citrate synthase: (**A**) complex I (NADH dehydrogenase); (**B**) complex II (succinate−cytochrome *c* reducatase); (**C**) complex III (ubiquinol−cytochrome *c* reductase); (**D**) complex IV (cytochrome *c* oxidase); (**E**) citrate synthase activity. The panels represent data collected from at least 10 separate experiments with different mitochondrial preparations. Error bar represents standard error about the mean ± SEM. ∗, $p < 0.05$ vs I/R.

ATP level and keep the mitochondria under energized condition. However, their degrees of protection are different for different complexes of ETC.

In the present study, citrate synthase activity was also measured. The enzyme citrate synthase exists in nearly all living cells and stands as a pacemaking enzyme in the first step of the citric acid cycle (or Krebs cycle). Here we have shown that in control-I/R heart, where the level of ATP production is low, citrate synthase activity is very low. Treatment with wines and their constituents increased the citrate synthase activity. Red wine and white wine are the most potent in restoring citrate synthase activity followed by reveratrol, tyrosol, and hydroxytyrosol. This result reconfirms our finding that wines and their constituents induced cardioprotection by restoring the activity mitochondrial ETC activity as well as mitochondrial integrity. A recent publication also showed that inhibition of citrate synthase activity after ischemia/reperfusion and NHE-1 inhibitor significanly increased citrate synthase and decreased MPTP and induced cardioprotection (*35*).

It is also well-known that swelling of mitochondria ruptures the outer mitochondrial membrane and releases pro-apoptotic proteins such as cytochrome *c*, Smac/Diablo, and apoptotic inducing factor (AIF) to activate energy-dependent apoptosis (*36*). Generally, opening of MPTP in the inner mitochondrial membrane causes collapse of membrane potential and swelling of mitochondria. The mitochondrial swelling assay showed that in control-I/R heart mitochondrial swelling is very high, but wine and their constituents reduced the mitochondrial swelling, presumably leading to the inhibition of cell death and induced cardioprotection. Consistent with these results, our studies with isolated working hearts demonstrated the reduction of cardiomyocyte apoptosis with both red and white wines and their components, which was also reflected in their abilities to reduce myocardial infarct size and improve ventricular function.

Activation of the PI 3-kinase/Akt signaling is known to provide cardiac protection against various stressors by preserving mitochondrial integrity and function. PI 3-kinase signaling has been shown to provide protection against I/R injury during preconditioning (*37*), and Akt activation has been shown to decrease apoptosis, as well as reduce infarct size and improve cardiac function after I/R (*38*). Furthermore, adenovirally infected rat hearts (*39*) with constitutively active Akt gene constructs confer protection against I/R injury. Akt has been shown to protect mitochondrial integrity and inhibit cytochrome *c* release following an apoptotic stimulus (*40*). However, the exact mechanism for Akt-mediated cardioprotection is still unclear, but it appears that Akt acts on multiple targets to provide its protective effects. For instance, preservation of mitochondrial integrity and function by Akt has been shown to be dependent on the presence of glucose and hexokinase. Akt was shown to preserve mitochondrial membrane potential in response to growth factor deprivation by increasing glucose

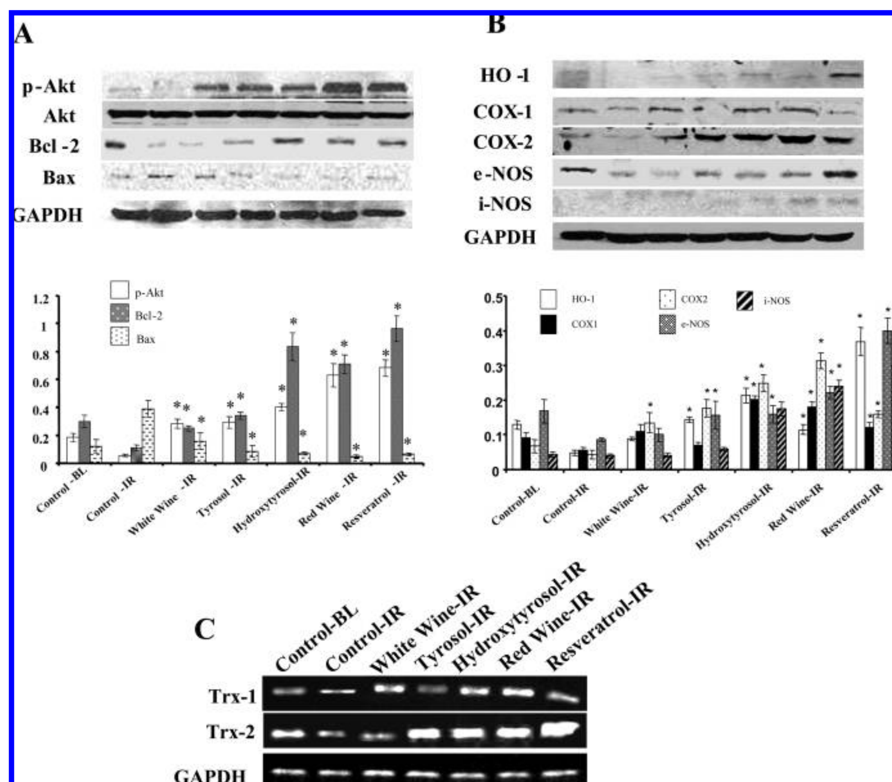

Figure 8. Western blot analysis of (**A**) p-Akt, Akt, Bax, and Bcl-2 proteins and (**B**) HO-1, COX-1, COX-2, e-NOS, and i-NOS in heart tissue obtained from control-BL, control-I/R and white wine-, tyrosol-, hydroxytyrosol-, red wine-, and resveratrol-treated rats. Panels are representative images of three different samples in each group. Values are mean ± SEM. $*$, $p < 0.05$ vs I/R. Representative photomicrographs are shown above the bar graphs. (**C**) Total RNA was extracted from left ventricular tissue, and mRNA transcripts of Trx-1 and Trx-2 were determined by RT-PCR from the isolated RNA of samples. RT-PCR of GAPDH was used as loading control. Representative images of three different samples in each group are shown, and each experiment was repeated at least three times.

transporter expression and glycolytic activity, resulting in greater substrate availability for mitochondrial electron transport (*41*). Akt also elevated mitochondrial hexokinase association and activity at the mitochondria (*40*). In addition, Akt has been shown to provide protection by phosphorylation and inactivation of the BH3-only protein Bad (*42*) and prevention of Bax translocation to the mitochondria (*43*), as well as induction of anti-apoptotic Bcl-2 proteins (*44*).

The results of the present study are consistent with these reports and demonstrate an induction of the expression of phospho-Akt and Bcl-2 protein. The results indicate the most significant activation of Akt and Bcl-2 for red wine and resveratrol, suggesting that resveratrol is the most potent compound for cardiomyocyte survival.

The Bcl-2 family of proteins play a central role in regulating apoptosis in the heart. The anti-apoptotic Bcl-2 proteins provide protection of mitochondria by acting on multiple targets. For instance, Bcl-2 has been shown to prevent permeabilization of the outer mitochondrial membrane by inhibiting activation of Bax/Bak (*44*) and to increase the calcium threshold for MPTP opening in heart mitochondria by blocking opening of the pore (*45*). Moreover, transgenic mice overexpressing Bcl-2 in the heart had fewer apoptotic cells, reduced infarct size, and improved recovery of cardiac function after I/R (*46*). During ischemia, electron transport and mitochondrial generation of ATP are inhibited, and the F_1F_0-ATPase runs in reverse, consuming glycolytically generated ATP (*47*). Interestingly, transgenic mice overexpressing Bcl-2 in the heart had a decreased rate in decline of ATP during ischemia as well as reduced acidification, suggesting that Bcl-2 provides protection by inhibiting the consumption of glycolytically generated ATP by the F_1F_0-ATPase (*46*). Moreover, elevated expression of Bcl-X_L by adenoviral gene transfer inhibited Bax translocation from the cytosol to the mitochondria, reduced cytochrome *c* release from mitochondria, and decreased apoptosis after I/R (*48*).

The results of our study demonstrated the induction of cardioprotective proteins such as iNOS, eNOS, COX-1, COX-2, HO-1, and transcripts Trx-1 and Trx-2 and by all of the treatments including red wine, white wine, resveratrol, tyrosol, and hydroxytyrosol. Increased nitric oxide content in the ischemic myocardium leads to cardiomyocyte survival (*49*). Activation of HO-1 and COX-2 is known to be mediated via NO in the ischemic myocardium. Whereas the abilities of red wine and resveratrol to induce NOS, COX-2, and HO-1 have been known for some time (*29, 50*), the ability of white wine to induce these enzymes is not known. It appears that resveratrol and red wine are the most effective agents to induce these phase II enzymes, as the same compounds are responsible for the most activation of nitric oxide. White wine and its active components, tyrosol and hydroxytyrosol, also activated eNOS, but not iNOS, as well as HO-1, COX-1, and COX-2. High expressions of Trx proteins have been shown to reduce cardiovascular dysfunction such as myocardial ischemia/reperfusion injury and ventricular fibrillation. Earlier studies have also shown that Trx may facilitate the induction of HO-1. Our study correlates the previous findings of showing cardioprotection by the activation of Trx-1 and Trx-2 for all treated wine and compound samples. Activation of thioredoxin, the most potent redox active protein, by both white and red wines raises the interesting possibility that the wines possess the capability of redox cycling.

In summary, the results of the present study document that white wines may possess cardioprotective ability comparable

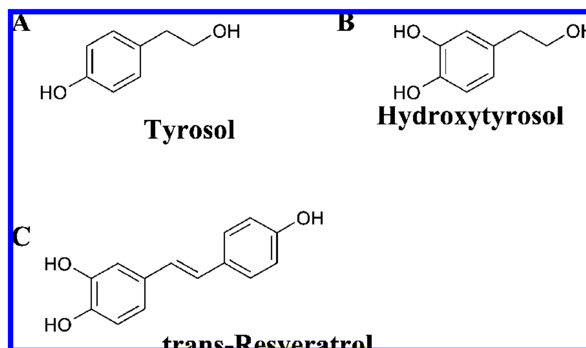

Figure 9. Structures for tyrosol, hydroxytyrosol, and *trans*-resveratrol.

to that of red wines if the wines are rich is tyrosol and/or hydroxytyrosol. The potential therapeutic target appears to be the mitochondria, where the wines can manipulate mitochondrial complexes. The similarities of mechanisms of action and molecular targets between red and white wines are probably due to structural similarities between resveratrol, tyrosol, and hydroxytyrosol (**Figure 9**). However, the potency of these polyphenols toward mitochondrial complexes and their abilities to generate survival signals are quite different. Finally, our study raises the interesting possibility of including white wine in the French paradox.

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