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Proanthocyanidin prevents myocardial ischemic injury in adult rats

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A Study Design
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E Manuscript Preparation
F Literature Search
G Funds Collection

Adem Guler[1AB], Mehmet Ali Sahin[1AB], Orhan Yucel[2AB], Mehmet Yokusoglu[3CE], Mehmet Gamsizkan[4D], Ertugrul Ozal[1F], Ufuk Demirkilic[1F], Mehmet Arslan[1G]

[1] Department of Cardiovascular Surgery, Gulhane Military Medical School, Ankara, Turkey
[2] Department of Thoracic Surgery, Gulhane Military Medical School, Ankara, Turkey
[3] Department of Cardiology, Gulhane Military Medical School, Ankara, Turkey
[4] Department of Pathology, Gulhane Military Medical School, Ankara, Turkey

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Summary

Background: Proanthocyanidin is a bioflavonoid known to have protective effect against oxidative injury. We investigated the cardioprotective effect of proanthocyanidin.

Material/Methods: Thirty-two *Rattus Norvegicus* rats were categorized equally as the control group (CG), proanthocyanidin group (PCG), ischemia group (IG) and proanthocyanidin-treated group (PCT). Rats in CG and IG were fed standard rat food and PCG and PCT were fed standard rat food plus proanthocyanidin (100 mg/kg/day twice a day by oral gavage) for 3 weeks. In CG and PCG the myocardial samples were prepared immediately, and in IG and PCT hearts were placed in transport solution and kept at 4°C for 5 hours, then prepared for evaluation. Malondialdehyde (MDA) level, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were measured.

Results: MDA levels were significantly higher in IG and PCT than in CG and PCG. The activity of SOD was significantly lower in IG and higher in PCG than in the other groups. The activity of GPx was significantly lower in IG than in the other groups. The activities of CAT were significantly lower in IG and PCT than in the other groups and were significantly lower in IG than PCT. Histopathologic evaluation revealed normal findings in CG and PCG. While ischemic injury was observed in IG, the content of muscle fibers was better preserved in PCT.

Conclusions: Proanthocyanidin may have a protective effect on myocardial ischemic injury.

key words: **proanthocyanidin • malondialdehyde • superoxide dismutase • catalase • glutathione peroxidase**

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Author's address: Mehmet Yokusoglu, Department of Cardiology, Gulhane Military Medical School, Gn. Tevfik Saglam Cad. 06018, Etlik, Ankara, Turkey, e-mail: myokusoglu@yahoo.com

Background

Diet has an important role in the pathogenesis of chronic diseases such as atherosclerosis, hypertension, type II diabetes, obesity and malignancies [1]. Epidemiological studies have demonstrated that diets rich in fruits and fruit seed extracts play a protective role against various cancer types and atherosclerosis [1]. Among these protectors, grape seed proanthocyanidin extract is a bioflavonoid through polyphenol compounds, with its known extensive pharmacological activity and therapeutic potential. Proanthocyanidin is found in large amounts in red wine and grape seed [2]. In a recent study conducted in northern France, prevalence of cardiovascular diseases was found to be lower in individuals who were intensive smokers and were on high cholesterol diet but consuming large amounts of red wine compared to those who did not consume large amounts of red wine, and this was introduced into the medical literature as the "French paradox" [3]. This was attributed to the effects of bioflavonoids, including proanthocyanidin found in red wine [3]. Bioflavonoids increase the antioxidant capacity in tissues and cells [4]. Proanthocyanidin exerts this effect by increasing the ability of scavenging hydroxyl and peroxy radicals [4]. They also provide postischemic cardiac protection by reducing oxygen-related free radical formation in the myocardium [5].

Free radicals and oxidative stress parameters are the most frequently used mediators to demonstrate cellular apoptosis during ischemia [6,7]. It has been found that reactive oxygen species play a significant role in the pathogenesis of stress-related damage in ischemic injury [8,9]. Reactive oxygen species can be directly cytotoxic or may pose potential risk for cell death by transforming into a free radical that reacts with macromolecules, DNA, proteins and lipids. Proanthocyanidin has been reported to have a protective effect against free radical-related oxidative injury in cultured macrophages [3].

In this experimental animal study we aimed to investigate the cardioprotective effect of proanthocyanidin on ischemic injury by simulating critical myocardial ischemia duration and conditions in humans by designing a model via removing hearts from the rats and keeping them in transport solution for 5 hours. The study protocol was reviewed and approved by the local ethics committee.

Material and Methods

Grape seed proanthocyanidin extract

Proanthocyanidin was obtained from a natural products market. Grape extract proanthocyanidin (*vitis vinifera* extract) was provided in a package of 30 capsules weighing 100 mg (Nutra Manufacturing Inc., Greenville, SC, USA). It contained proanthocyanidin, herbal cellulose, dicalcium phosphate, stearic acid, and magnesium stearate.

Animals and laboratory

All procedures in the present study were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA). Thirty-two male *Rattus Norvegicus* rats (Gulhane Military Medical School, Experimental Research and Animal Laboratory, Ankara) aged 17–19 weeks with body weight 320–340 g were used. They were housed in a quiet, temperature- and humidity-controlled room (22±2°C and 60±5%, respectively) in which a 12-h light/dark cycle was maintained (07:00–19:00 h light) as previously described [10,11]. Rats were housed in metal cages in pairs 3 days before the study. Rats were fed with easily accessible standard rat food (Bil-Yem Gida Sanayi Tic. Ltd. Sti., Yenikent, Ankara) and tap water.

Groups and treatments

Rats were randomly allocated into 4 groups as the control group (CG), proanthocyanidin group (PCG), ischemia group (IG) and proanthocyanidin-treated group (PCT), each consisting of 8 rats. Rats in the CG and IG were fed standard rat food for 3 weeks. Rats in the PCG and PCT were fed rat food plus proanthocyanidin (100 mg/kg/day twice a day by oral gavage) for 3 weeks.

Sampling

All rats were sacrificed by a lethal dose of ketamine and xylazine at the end of 3 weeks. To access the heart, median sternotomy was performed. After the blood in the circulation was drained by dissecting the inferior vena cava, the circulatory system was irrigated by Plegisol solution through an aortic cannula. The hearts were removed after cardiac arrest. Samples were immediately obtained from the left ventricles of rats in CG and PCG for histopathological and biochemical evaluation. For histopathological evaluation, samples were fixed by 10% formaldehyde solution. For biochemical evaluation, samples were frozen in liquid nitrogen, and immediately sent to the freezer (–80°C) after being placed in polypropylene tubes. Left ventricle tissue samples of rats in IG and PCT were placed in transport solution (0.09% NaCl) and kept at 4°C for 5 hours. Five hours after sacrificing the rats, sampling for histopathological and biochemical evaluations in these hearts were also performed as described above for CG and PCG.

Histopathological evaluation

Following fixation and routine processing, 4 μm-thick sections were obtained from left ventricle tissue samples. Sections were stained with hematoxylin-eosin (HE). All samples were evaluated by a blinded pathologist for intercellular edema, inflammatory cell infiltration, muscle fiber degeneration, intracytoplasmic vacuolization, hemorrhage and mast cell degranulation.

Biochemical evaluation

Malondialdehyde (MDA) level, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzyme activities were measured as oxidative stress parameters in tissue samples stored for biochemical evaluation. MDA level, SOD, CAT and GPx activity levels were measured as previously described [12].

Determination of MDA level

Before the preparation of left ventricle tissue for analysis, KCl solution to be used in the analysis was prepared.

Table 1. Comparison of descriptive statistics results between study groups.

Parameters	Group	N	Min-Max	Median	Mean±SD	Test statistics	p
MDA (nmol/mL)	CG	8	3.32–3.74	3.52	3.53±0.14	X^2=23.698	<0.001
	PCG	8	1.46–4.56	3.19	3.01±1.09		
	IG	8	6.93–11.23	8.46	9.04±1.63		
	PCT	8	5.76–9.81	8.06	7.80±1.47		
SOD (U/g)	CG	8	84.03–96.73	91.12	90.38±5.17	X^2=26.045	<0.001
	PCG	8	101.15–110.67	105.42	105.91±3.20		
	IG	8	51.50–64.28	58.90	58.14±4.17		
	PCT	8	87.11–104.79	95.60	95.95±6.32		
GPx (U)	CG	8	20.88–23.14	21.92	22.01±1.01	X^2=17.743	<0.001
	PCG	8	20.44–24.50	22.64	22.47±1.30		
	IG	8	14.32–17.90	16.36	16.11±1.11		
	PCT	8	18.18–25.56	22.24	21.87±2.49		
CAT (kU/g)	CG	8	6.05–7.31	6.73	6.68±0.47	F=50.314	<0.001
	PCG	8	6.09–6.97	6.53	6.52±0.30		
	IG	8	4.06–5.02	4.61	4.61±0.39		
	PCT	8	4.90–5.92	5.38	5.41±0.39		

Min – minimum; Max – maximum; SD – standard deviation; CG – control group; PCG – proanthocyanidin group; IG – ischemia group; PCT – proanthocyanidin treated group; MDA – malondialdehyde, SOD – superoxide dismutase; GPx – glutathione peroxidase; CAT – catalase.

Left ventricle tissue was then homogenized. Reagents required for measurement of MDA level in the left ventricle tissue were prepared. Homogenized left ventricle samples of 0.2 mL were placed into 10 mL polypropylene tubes in icy conditions and then 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid, and 0.6 mL of distilled water were added (total volume 5 mL). The tubes were kept in a boiling water bath at 95°C for 1 hour, and then 1 mL of distilled water and 5 mL of n-butanol were added (total volume 10 mL). The tubes were centrifuged at 4000 rpm for 10 minutes, the supernatant was transferred into 3 mL cuvettes by a pipette and their optical absorbance at 532 nm was measured against a sample blank. The concentrations of MDA in the samples were calculated directly from the calibration curve in nmol/mL.

Determination of SOD activity

Required reagents were prepared. In order to prepare 500 mL of standard SOD solution phosphate buffer, 0.6805 g of KH_2PO_4 was weighed, transferred into a 500 mL balloon flask and dissolved in distilled water. Total volume was completed by adding 15 mL of 0.2 M NaOH and the pH was adjusted to 7 by o-phosphoric acid or NaOH.

Preparation of the reaction media

For 40 mL of mixture, 442.6 mg 3-(cyclohexylamine)-1-propanesulfonic acid and 13.99 mg ethylenediaminetetraacetic acid were weighed and completed to 40 mL, and pH was adjusted to 10.2 by o-phosphoric acid or NaOH. Then, 0.348 mg of xantine Na and 0.506 mg of iodonitrotetrazolium were added into this solution. Xantine oxidase (80 U/I) was prepared, and 0.2 mL of 10% homogenates was taken by a 0.1 mL pipette and 3.9 mL of buffer was added. Thus, the solution was 400 times diluted. Then, 25 µL tissue homogenate, 850 µL of substrate mixture and 125 µL of xantine oxidase were added into 1 mL cuvettes, mixed and absorbances at 505 nm were measured against a sample blank by ultraviolet spectrophotometer at the 30th and 210th seconds. SOD activity levels were calculated. The same procedure was performed for all tissues analyzed.

Determination of GPx activity

Reagents required for measurement of GPx activity level were prepared. Then, 0.1 mL of 10% homogenates was pipetted, 1.6 mL of dilution was added and vortexed, and 990 µL of reaction mixture, 10 µL diluted homogenate and 10 µL t-butyl hydroperoxide were added into 1 mL cuvettes. Absorbances were measured for 3 minute durations with 30 second intervals by ultraviolet spectrophotometer and recorded. GPx activity levels were calculated. The same procedures were performed for all tissue samples analyzed.

Determination of CAT activity

Left ventricle tissue homogenates were prepared. Activity of CAT in tissue homogenates was measured at 25°C and substrate H_2O_2 decomposition rate was observed spectrophotometrically for 30 seconds at 240 nm. Activity was expressed in kU/g.

Table 2. Post-hoc test results for malondialdehyde, superoxide dismutase glutathione peroxidase and catalase parameters.

Parameters	Group	Group	Z	p
MDA	CG	PCG	0.210	0.878
		IG	3.363	**<0.001**
		PCT	3.363	**<0.001**
	PCG	IG	3.361	**<0.001**
		PCT	3.361	**<0.001**
	IG	PCT	1.260	0.234
SOD	CG	PCG	3.361	**<0.001**
		IG	3.361	**<0.001**
		PCT	1.680	0.105
	PCG	IG	3.361	**<0.001**
		PCT	2.941	**0.002**
	IG	PCT	3.361	**<0.001**
GPx	CG	PCG	0.843	0.442
		IG	3.363	**<0.001**
		PCT	0.000	1.000
	PCG	IG	3.361	**<0.001**
		PCT	0.315	0.798
	IG	PCT	3.361	**<0.001**
CAT	CG	PCG	0.16	1.000
		IG	2.07	**<0.001**
		PCT	1.27	**<0.001**
	PCG	IG	1.91	**<0.001**
		PCT	1.11	**<0.001**
	IG	PCT	–0.80	**0.002**

CG – control group; PCG – Pproanthocyanidin group;
IG – ischemia group; PCT – proanthocyanidin treated group;
MDA – malondialdehyde; SOD – superoxide dismutase;
GPx – glutathione peroxidase; CAT – catalase.

Statistical analysis

All statistical analysis and calculations were performed using SPSS for Windows Version 15.00 (SPSS Inc, Chicago, IL, USA). Normal distribution of the study parameters was tested both graphically and statistically by Shapiro-Wilk test. It was noted that except for CAT, MDA, SOD and GPx were not normally distributed. Descriptive statistics are presented as mean ± standard deviation (SD) and median. Kruskal-Wallis variance analysis was performed for comparison of MDA, SOD, GPx parameters in the study groups. Comparisons between paired groups were performed by Bonferroni-corrected Mann-Whitney U test. One-way ANOVA was performed for between-group comparison of CAT parameters. Bonferroni post-hoc test was used to determine the source of difference. Significance level was accepted as a P value below 0.05.

Results

Biochemical evaluation

There was no significant difference between CG and PCG in terms of oxidative stress parameters, including MDA level and SOD, CAT, GPx activity levels. While MDA level was elevated in the IG, activities of SOD, CAT and GPx were reduced. While proanthocyanidin treatment decreased the amount of ischemia-related MDA elevation to a certain level, it increased the level of other oxidative stress parameters, including SOD, CAT and GPx activity. Comparison of results between the study groups is presented in Table 1.

Significant differences were found between all groups in terms of MDA, SOD, GPx and CAT parameters ($p<0.001$). For non-normally distributed parameters, including MDA, SOD and GPx parameters, Bonferroni-corrected Mann-Whitney U test was performed to determine the source of difference. Post-hoc test results are presented in Table 2.

While the MDA levels of IG and PCT were significantly different compared to CG, the difference was not significant between CG and PCG. MDA levels of IG and PCT were significantly different compared to PCG, whereas there was no significant difference between MDA levels of IG and PCT. The highest MDA level was measured in IG and the lowest was measured in PCG.

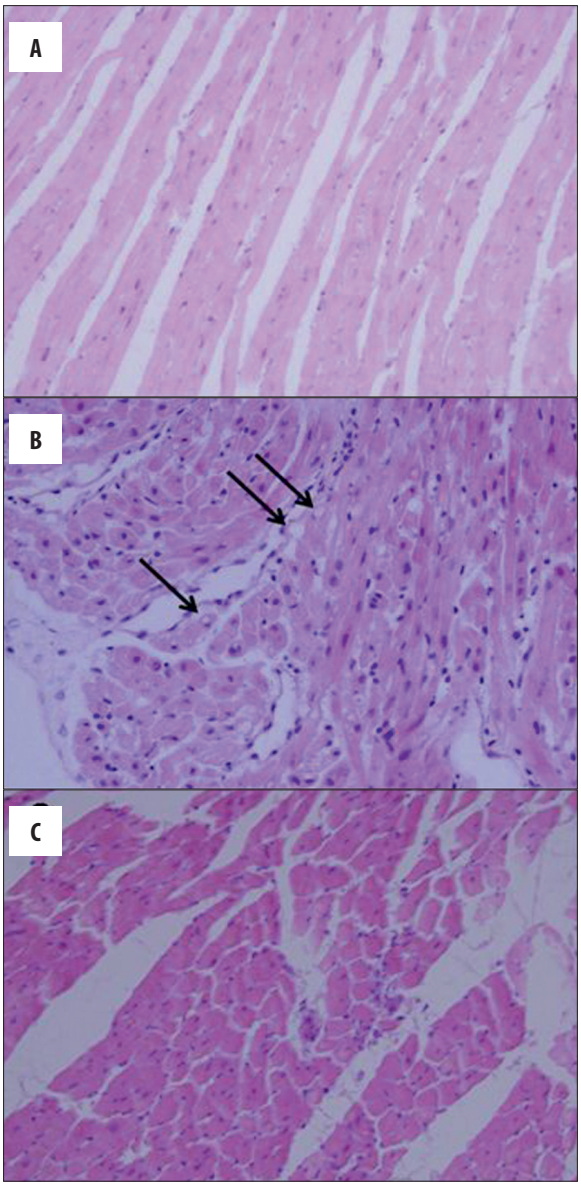
While the SOD activities of CG and PCT were similar and there was no significant difference between these groups, the SOD activities of the remaining groups were all significantly different from each other. The highest SOD level was observed in PCG and the lowest level in IG.

The GPx activities of CG, PCG and PCT were significantly different compared to IG. No significant difference was found between the remaining groups. While GPx level was lowest in IG, it was similar in other groups.

No significant differences in CAT levels were found between CG and PCG, while a significant difference was noted between all other groups. Lowest CAT level was measured in IG, and CAT level was highest in CG.

Histopathological evaluation results

Myocardial fibrils were within normal range in CG and PCG (Figure 1A). In the IG, myofibrils were relatively indistinct in the muscle fibers that had picnotic-dark or pale nuclei and more acidophilic cytoplasm. Intracytoplasmic vacuoles were observed in some muscle fibers (Figure 1B). Muscle fiber organization was impaired and they were separated from each other due to intracellular edema in certain regions (Figure 1C). Inflammatory cell infiltrations were seen in some areas (Figure 1D). Degranulation of mast cells was rarely noted in the connective tissue. In PCT the organization of muscle fibers was better preserved compared to the IG; there were no vacuolar changes or necrosis (Figure 1E).



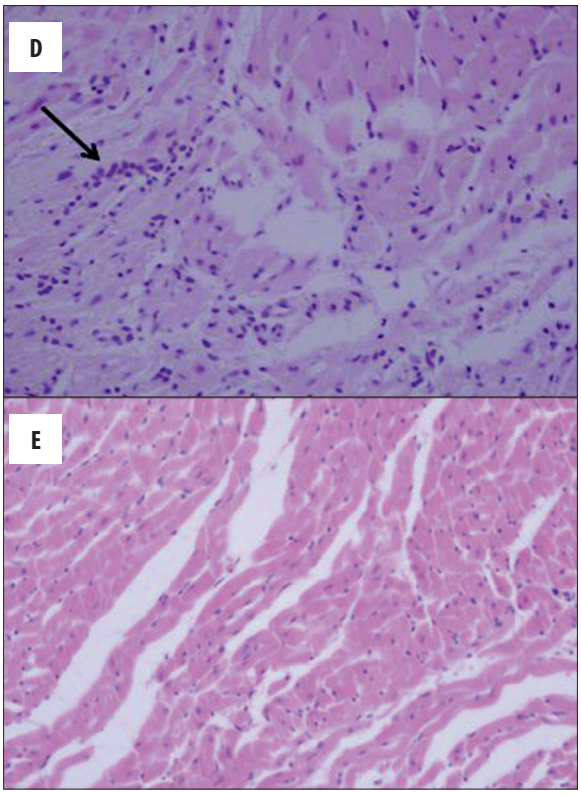


Figure 1. (**A**) Normal myocardial fibers in control group (He ×100). (**B**) Light vacuolar changes (arrow) in ischemia group (HE ×200). (**C**) Focal myocardial fiber degeneration in ischemia group (HE ×100). (**D**) Inflammatory cell infiltration (arrow) among the myocardial fibers in ischemia group (HE ×200). (**E**) Muscle fibers in treatment group are better preserved to compare to the ischemic ones (He ×100).

Discussion

The main finding of this study is the beneficial effect of proanthocyanidin on ischemic injury of myocardium, which is shown histopathologically.

If the ischemic period is prolonged, depletion of high energy phosphate stores, reduction of intracellular pH, accumulation of metabolic degradation products and cell membrane damage are observed. As a result of the depletion of high energy phosphate storage during myocardial ischemia, an excessive amount of Ca^{+2} is accumulated in cytoplasm and mitochondria of the cell and it exerts toxic effects. Failure of pumps and storage mechanisms occur due to energy depletion, and increased levels of Ca^{+2} leads to cell injury by activating phospholipases and proteases and by increasing free radicals and fatty acids formation [13]. On histological evaluations, myocardial fibrils exposed to ischemia are stained more acidophilic due to reduced intracellular pH level [14].

In this study, intensely acidophilic stained muscle fibers with relatively indistinctive myofibrils were observed in the histological evaluation of the ischemic area of the IG. Muscle fibers organization was impaired and separations were noted in certain regions. In the PCT, there were occasional muscle fibers with acidophilic cytoplasm and picnotic nuclei; however, myofibril structure in the fibers was distinct. The better histological findings in the PCT might be associated with the protective effect of proanthocyanidin.

Several recent studies showed that flavonoids have favorable effect on ischemia/reperfusion injury on brain, liver, kidney and eye via their antioxidant effect [15–18]. It has also been demonstrated in several studies designed to reduce ischemic myocardial injury that diet rich in fruit seed extract plays a protective role against cardiovascular diseases [1]. Among these protectors, grape seed proanthocyanidin extract is a bioflavonoid through polyphenol compounds with its known extensive pharmacological activity and therapeutic potential. Proanthocyanidin is an antioxidant and prevention of free oxygen radical formation is the most prominent function of antioxidants. Antioxidant enzymes are depleted in myocardial ischemia. Hemodynamically, it increases aortic and coronary flow and reduces left ventricle pressure [2]. Pataki et al. [19] reported that proanthocyanidin could be used in routine heart surgery and heart transplantation owing to its positive effects on functional

improvement of the heart and its reducing effect on potential post-ischemia arrhythmias. Our findings were in agreement with previous studies and we believe that proanthocyanidin administration will prolong the ischemia duration of the heart, and may improve postoperative cardiac functions.

Many agents, such as vitamin C, vitamin E, β-carotene, L-carnitine, caffeic acid, lazaroid, prunus cerasus (sour cherry) have been used to increase resistance to ischemia [20]. These agents may contribute to the recovery of the myocardium following ischemia by reducing free radical formation or exerting a scavenging effect. Proanthocyanidin increases the antioxidant capacity in tissues and cells [2–4]. They also provide post-ischemic cardiac protection by reducing oxygen-related free radical formation in the myocardium [5,9]. *In vitro* studies have shown that proanthocyanidin exerts this potential effect by scavenging peroxy and hydroxyl radicals. In our experimental study, an increase in MDA level and decrease in SOD, CAD and GPx activities in the IG was observed. While proanthocyanidin treatment decreased the amount of myocardial ischemia-related MDA elevation to a certain level, it increased the activity levels of other oxidative stress parameters, including SOD, CAT and GPx. This result might indicate that proanthocyanidin is a myocardial protector. Nevertheless, further studies are needed to determine the optimal dose, duration and timing of treatment. Although it was not statistically significant, we considered that approximately 1.20 nmol/mL of difference in MDA level between IG and PCT is clinically important. Lack of this statistical significance might be due to small sample size.

Another pathway for proanthocyanidin to exert its effect on myocardial protection is via its effect on intracellular calcium concentrations. Proanthocyanidin leads to reduction of ionized calcium levels by interacting with intracellular calcium ions [19]. This mechanism should be investigated in future studies evaluating the effectiveness of proanthocyanidin.

Conclusions

The results of the present study demonstrate that proanthocyanidin may have a protective effect on myocardial ischemia and that this function can be used to prolong the ischemic period. Our results need to be confirmed and supported by further clinical and experimental studies. Proanthocyanidin might have exerted this protective effect both by its free radical scavenging effect and myocyte cell membrane stabilization effect. We believe that our finding of less injury noted in PCT compared to IG, according to our histological and biochemical results, suggests a myocardial protective effect of proanthocyanidin; however, these results were obtained from an animal study. Adaptation of the results to human clinical practice must be implemented after long-term randomized clinical studies on humans have been conducted.

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