

The effects of resveratrol treatment on caveolin-3 expression and Na⁺/K⁺ ATPase activity in rats with isoproterenol-induced myocardial injury

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ABSTRACT

OBJECTIVE: The present study aims to investigate the therapeutic effects of resveratrol (RES) on isoproterenol (ISO) induced myocardial injury rat model.

METHODS: Catecholamine-induced heart damage was induced by ISO treatment for 30 days. The rats were divided into four groups as follows: the control group received saline, the ISO group received 5.0 mg/kg ISO, the RES group received 10 mg/kg RES, and the ISO-RES group received 10 mg/kg RES and 5 mg/kg ISO treatments for 30 days. Following echocardiographic measurements and body weight recorded, the rats were decapitated. Plasma and cardiac tissue samples obtained by decapitation were analyzed using biochemical, histopathological, molecular and immunohistochemical methods.

RESULTS: In the ISO group, Na⁺/K⁺ ATPase activity and ATP content, GSH, and caveolin-3 levels were low. LDH, CK and lysosomal enzyme activities, MDA level, and MPO activity were found to be high. It was determined that GSH and MDA levels and MPO, Na⁺/K⁺ ATPase activity, ATP content caveolin-3 levels changes that arose from ISO treatment were suppressed by RES treatment.

CONCLUSION: RES treatment has ameliorated all the functional and biochemical parameters. The results obtained in this study suggest that RES is a promising supplement against catecholamine exposure as it improves antioxidant defense mechanisms in the heart. In the light of above-mentioned data, RES can be assumed as a promising agent in ameliorating the oxidative injury of the myocardium.

Keywords: Caveolin-3; heart failure; isoproterenol; lysosomal enzymes; resveratrol.

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Coronary artery disease (CAD) and subsequent heart failure (HF) are among the major health problems in this century. As an adaptive mechanism, catecholamines play an important role in stress-induced neurohumoral activation during both HF and coronary ischemia. In addition, prolonged exposure to catecholamine causes myocardial damage [1–3]. Therefore, in our study, a myocardial injury rat model was created by administering isoproterenol (ISO), which is a catecholamine. In particular, in ISO-induced myocardial injury rat model studies, it has been reported that increased ROS-induced intracellular lysosomal enzymes and intracellular antioxidant enzyme depletion in cardiomyocytes are associated with excessive calcium overload [4–6].

In the literature, consumption of resveratrol (RES, 5-(e)-2-(4-hydroxyphenyl) ethenylbenzene-1,3-diol), a naturally occurring phytochemical polyphenolic compound, has been reported to reduce mortality from coronary heart disease [7–9]. In addition, it is emphasized that the strong antioxidant activity of RES shows other therapeutic and protective actions, such as anti-inflammatory properties [7]. In our previous study, we demonstrated that RES treatment reduces cardiotoxicity and reduces the severity of cardiac dysfunction in rats receiving doxorubicin [8]. Similar protective effects of RES in hypertension were also shown in the study of Toklu et al. [9], where RES improved cardiovascular function by promoting antioxidant defense mechanisms. Accordingly, in the present study, we aimed to clarify whether RES can improve cardiovascular function and alleviate the severity of ISO-induced myocardial injury.

MATERIALS AND METHODS

Animals, experimental procedure and treatments

Approval was obtained from the Marmara University Animal Care and Use Committee (43.2007.mar.) for all experimental protocols. Wistar albino rats (200–250 g; male rats) were randomly divided into four groups for specific treatments for 30 days. Group 1- Control group treated with saline; Group 2- RES group was injected with RES (10 mg/kg/i.p) once daily [8]; Group 3- ISO group received isoproterenol hydrochloride (5.0 mg/kg/i.p) once daily [10]; Group 4- ISO-RES group, treated daily RES (10 mg/kg/i.p) and ISO (5.0 mg/kg/i.p). RES and ISO were supplied from Sigma (St. Louis, MO).

At the beginning of the experiment, body weight (BW) of all rats in the experimental group was recorded.

On the 30th day of the treatments, transthoracic echocardiographic measurements were performed. After the decapitation, heart weight (HW) was used to calculate the hypertrophy index (HI: HW/BW ratio; mg/g) [10].

Echocardiography

According to the guideline published by the American Society of Echocardiography, echocardiographic examinations were performed during systole and diastole under ketamine anesthesia [11]. The measurements of the interventricular septal thickness (IVST), left ventricular diameter (VDL) and posterior wall thickness (PWTL) were performed. In addition, ejection fraction (EFr), fractional shortening (FrS) and left ventricular mass (VML) were calculated using the formula [12].

Blood Assay

After decapitation, blood was collected and centrifuged at 3000 x g for 10 min; plasma creatine kinase (CK), lactate dehydrogenase (LDH), lysosomal enzymes (β -D-glucuronidase, β -galactosidase, β -D-N-acetylglucosaminidase, acid phosphatase (APs) and cathepsin-D) were analyzed using Opera Technicon *Auto Analyzer*® II.

Tissue Assays

Following decapitation, cardiac tissue samples were taken and stored at –80 °C for tissue analysis.

Measurement of the GSH and MDA Levels, and MPO Activity

The levels of GSH and MDA expressed as μ mol/g and nmol/g in the tissue samples were measured with the method of Beutler [13] and Buege and Aust [14], respectively. Additionally, the activity of MPO expressed as U/g tissue was measured by the procedure reported by Hillegass et al. [15].

Measurement of Collagen Content

The collagen content of the tissue samples was determined by the method described by López-De León and Rojkind [16].

Measurement of the Na⁺/K⁺ ATPase Activities and ATP Content

The Na⁺/K⁺ ATPase activity measurements of the tissues expressed as μ mol Pi mg⁻¹ protein h⁻¹ were eval-

uated by the Reading and Isbir method [17], while the ATP levels expressed as nmol/mg were evaluated using the bioluminescent test kits (Sigma-Aldrich, St. Louis, Missouri, USA).

Western Blotting

Extracted tissue lysate was transferred to nitrocellulose a membrane in transferring buffer (9.2g Tris, 43.2 g glycine, 600 ml methanol 2.4 L ddH₂O) to provide detachment from 12% SDS-PAGE gel. After incubation with a primary cave-3 monoclonal antibody (\approx 20 kDa; cat no: 610420, BD Transduction Lab.) during 14 h, and with secondary horseradish peroxidase-conjugated antibody (cat no: 554002, BD Transduction Lab) during one h, imaging was provided with chemiluminescence western detection system. β -actin was used for normalization of all membranes.

Histopathological Analysis

Tissue samples were fixed to 10% formaldehyde solution for longer than three hours, washed in tap water, and dehydrated within 70, 90, 96 and 100% concentration of alcohol solutions (15, 15, 30 and 30 min respectively), kept in a 100% toluene solution for 30 min. After toluene treatment, tissues were kept at 60 °C paraffin for one night and then fixed in the paraffin block. Sections at 5–4 μ m thickness were cut from paraffin blocks on slides. The sections were kept in the toluene solution for two hours for deparaffinization. Then, sections were hydrated in decreasing alcohol solutions (100, 90, and 70% for two min) and in distilled water for two min. The hydrated sections were stained with hematoxylin for 15 min and then washed in tap water to provide a purple-blue color. After eosin staining for 5 min, the sections were dehydrated in increasing concentration of alcohol solutions (70, 90, 96% during 2 min and 10 min 100%). Finally, tissue sections were mounted to examine under the light microscope (Olympus BH 2, Tokyo, Japan).

Immunohistochemistry

6- μ m-thick cut sections were taken from the deparaffinized process described above and rehydrated positively charged slides for immunohistochemical assay. For antigen retrieval, the sections were kept in Antigen Unmasking Solution (Vector Laboratories) in 90 °C for 20 min and incubated with anti-cav-3 antibody (1:500 dilution) (ab150077, Abcam, Cambridge, UK) for one night. It was incubated all sections with secondary an-

TABLE 1. Body weight, heart weight, and heart/body weight ratio values in the control, RES, ISO, and ISO-RES groups

	Control	RES	ISO	ISO-RES
BW (g)				
t_2	219 \pm 2.9	220 \pm 1.9	219 \pm 3.1	215 \pm 3.9
t_1	269 \pm 4.9*	274 \pm 4.5*	231 \pm 4.2 ^{&}	262 \pm 6.3* ⁺⁺
HW (mg)	578 \pm 14.1	580 \pm 14.6	830 \pm 45.9 ^{&}	643 \pm 27.6 ⁺⁺
HW/BW ratio (mg/g)	2.18 \pm 0.06	2.11 \pm 0.05	3.51 \pm 0.27 ^{&}	2.51 \pm 0.16 ⁺

Each group consists of eight rats. *: $p < 0.001$: vs t_1 value; +: $p < 0.01$; ++: $p < 0.001$: vs ISO group; &: $p < 0.001$: vs control group; RES: Resveratrol (10 mg/kg/i.p); ISO: Isoproterenol (5 mg/kg/i.p); HW: Heart weight; BW: Body weight; t_1 : Beginning of experiments; t_2 : 30th (the last) day of experiments.

tibody Alexa 488 (1:1000 dilution) (ab2912, Abcam, Cambridge, UK) and Cy3-conjugated donkey anti-mouse IgG antibody (1:1000 dilution) (ab150077, Abcam, Cambridge, UK) during one h. Finally, the sections were examined using a Zeiss LSM 5 PASCAL laser scanning confocal microscope.

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) and Tukey's test for the advanced analysis (GraphPad software; Prism 3.0; GraphPad Software, San Diego, CA, USA). All data were expressed as means \pm SEM. A value of $p < 0.05$ was considered significant.

RESULTS

At the beginning of the experiment (t_1), no significant difference was found between the groups in BW measurements (p : n.s; Table 1). On the 30th day of the follow-up period (t_2), BWs significantly increased in the whole of the groups, unlike the ISO group ($p < 0.001$; Table 1). We found that the increase in HW and HW/BW ratio in the ISO group was alleviated by RES ($p < 0.001$).

In the ISO group compared to the control group, the relative wall thickness (WT_R), left ventricular end-diastolic ($VEDD_L$) and end-systolic dimensions ($VESD_L$) increased; PWT, percentage FrS, and EF_r were found to be decreased ($p < 0.05$ – 0.001 ; Table 2). However, in the RES-treated ISO group, the increments in the WT_R and VDL dimensions were reduced ($p < 0.01$), and PW, the percent of FrS and EF_r measurements

TABLE 2. The transthoracic echocardiographic measurements of the control, RES, ISO, and ISO-RES groups

	Control	RES	ISO	ISO-RES
IVST (mm)	2.09±0.11	2.10±0.15	2.23±0.13	2.01±0.17
PWT _L (mm)	2.30±0.11	2.34±0.09	1.73±0.12**	2.20±0.10 ⁺
WT _R	0.60±0.03	0.64±0.05	0.81±0.04*	0.63±0.05 ⁺
VESD _L (mm)	2.49±0.14	2.68±0.16	3.56±0.14***	2.92±0.12 ⁺
VEDD _L (mm)	4.14±0.11	4.04±0.12	4.99±0.21**	4.35±0.14 ⁺
EFr (%)	81.3±2.9	78.8±1.5	63.3±2.9***	76.7±3.2 ⁺⁺
FrS (%)	40.2±2.2	39.5±1.9	22.5±1.1***	34.2±2.2 ⁺⁺

Each group consists of eight rats. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; compared to control group. +: $p < 0.05$; ++: $p < 0.01$; compared to ISO group. RES: Resveratrol (10 mg/kg/i.p); ISO: Isoproterenol (5 mg/kg/i.p); IVS: Interventricular septal thickness; PWT_L: Left ventricular posterior wall thickness; WT_R: Relative wall thickness; VESD_L: Left ventricular diameter in systole; VEDD_L: Left ventricular diameter in diastole; EFr: Ejection fraction; FrS: Fractional shortening.

augmented ($p < 0.001$). IVST remained unchanged in all groups (Table 2).

Plasma LDH, β -D-glucuronidase, β -D-N acetylglucosaminidase, APs, β -galactosidase, cathepsin-D, and CK activity which can be assumed to be the result of generalized tissue injury showed an elevation in the ISO group compared to the control group ($p < 0.05$ – 0.001). However, it was determined that RES treatment reversed the increase in all these enzyme activities ($p < 0.001$; Table 3).

When GSH levels of the tissue were examined, it was determined that there was a decrease in the ISO treatment group compared to the control group ($p < 0.01$), whereas, in the RES-ISO group, GSH content of the tissue was maintained similar to the control group ($p < 0.05$, Fig. 1A). Tissue MDA levels elevated with ISO treatment according to the control group ($p < 0.001$), while RES treatment significantly reduced the increase in ISO-induced MDA ($p < 0.01$; Fig. 1B). MPO activity, indicating neutrophil infiltration and subsequently oxidative stress, was significantly higher in the ISO group as compared to the control group ($p < 0.001$; Fig. 1C). However, in the ISO-RES group, cardiac MPO activity significantly depressed ($p < 0.01$) and was similar to the MPO activity of the control group. Cardiac collagen content, which indicated increased fibrotic activity increased significantly in the ISO group compared to the control group ($p < 0.001$; Fig. 1D). RES treatment, when added to ISO, significantly reduced cardiac collagen content ($p < 0.01$) back to control levels.

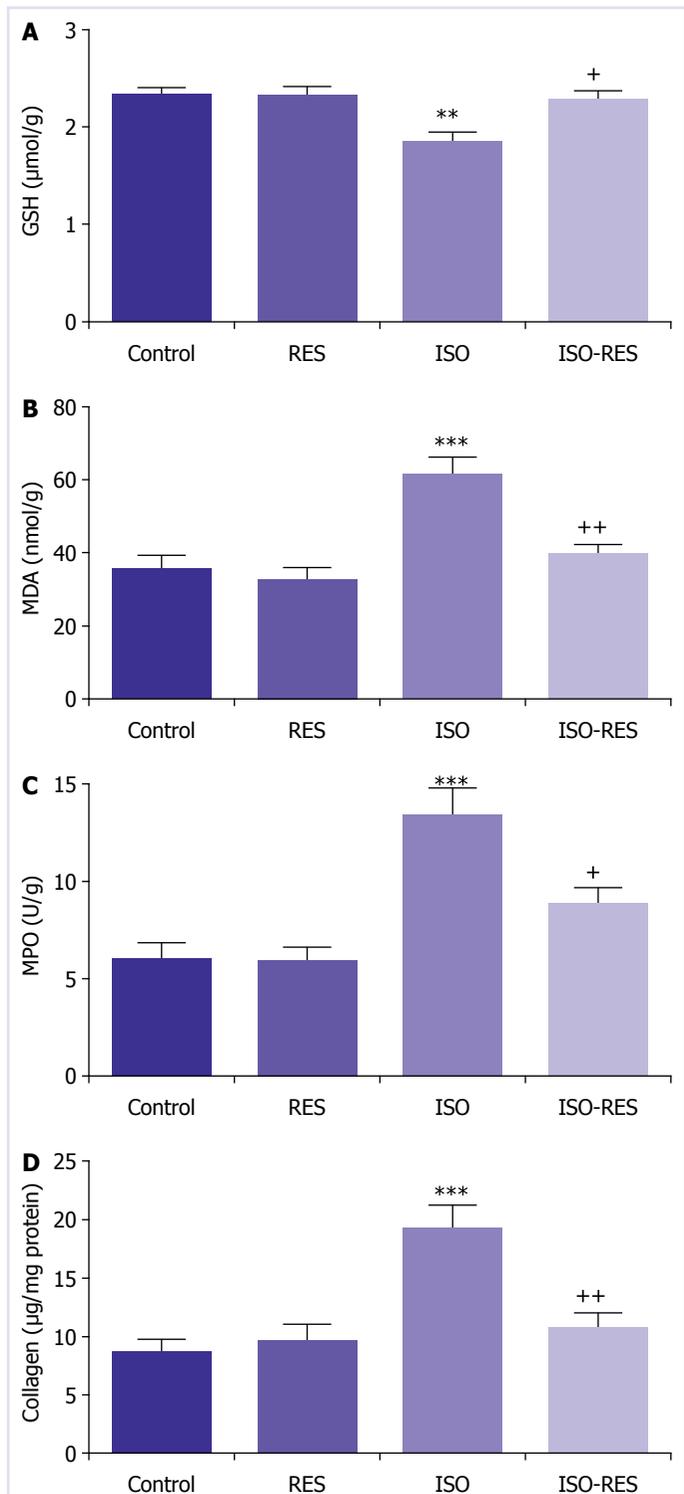


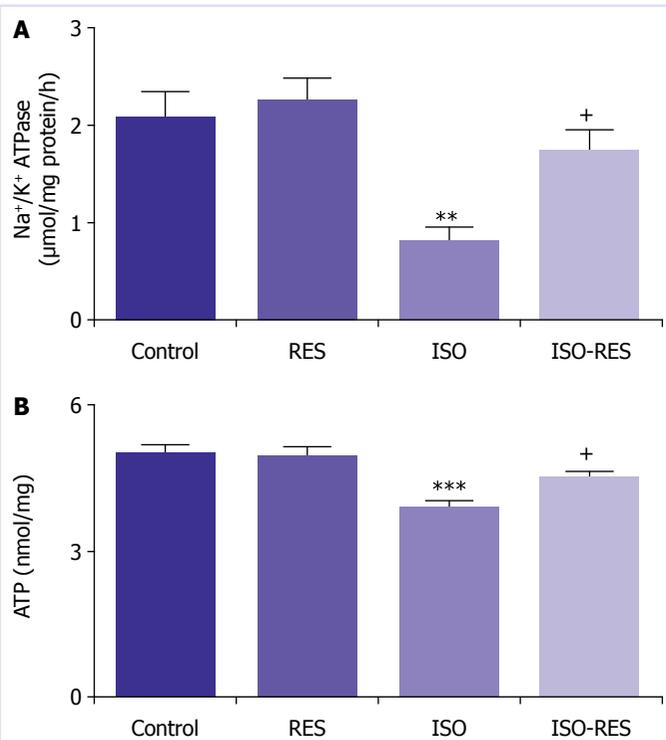
FIGURE 1. (A) Glutathione (GSH), (B) Malondialdehyde (MDA), (C) Myeloperoxidase (MPO) and (D) Collagen levels in the heart tissue of the control, RES, ISO, ISO-RES groups. Data are presented as means±SEM. For each group n=8.

** $p < 0.01$; *** $p < 0.001$ versus control group; + $p < 0.05$; ++ $p < 0.01$ versus ISO group; RES: Resveratrol (10 mg/kg/i.p); ISO: Isoproterenol (5 mg/kg/i.p).

TABLE 3. The plasma lactate dehydrogenase (LDH), creatinine kinase (CK), lysosomal enzymes activity (β -D-glucuronidase, β -galactosidase, β -D-N-acetylglucosaminidase, Aps, and cathepsin-D of control, RES, ISO, and ISO-RES groups

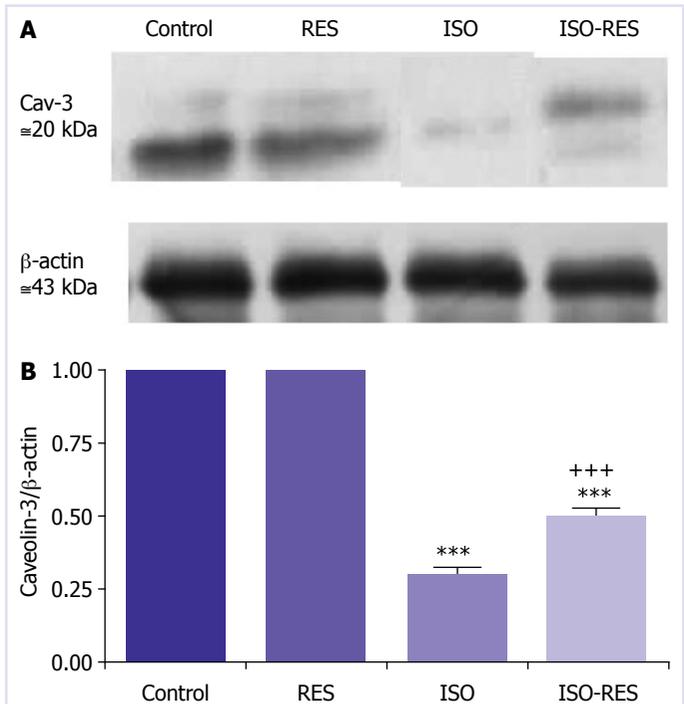
	Control	RES	ISO	ISO-RES
LDH (U/L)	1465 \pm 76	1506 \pm 102	3187 \pm 179**	1977 \pm 153+++
CK (U/L)	1687 \pm 124	1682 \pm 138	7162 \pm 316**	2537 \pm 219*,+++
β -D-glucuronidase (U/ml)	0.30 \pm 0.03	0.27 \pm 0.02	0.58 \pm 0.05**	0.32 \pm 0.04+++
β -galactosidase (U/ml)	0.40 \pm 0.03	0.35 \pm 0.04	0.66 \pm 0.09*	0.43 \pm 0.05*,+
β -D-N-acetylglucosaminidase (U/ml)	0.61 \pm 0.05	0.052 \pm 0.06	1.17 \pm 0.09**	0.62 \pm 0.08+++
APs (U/ml)	1.70 \pm 0.08	1.69 \pm 0.09	2.84 \pm 0.19**	1.84 \pm 0.13+++
Cathepsin-D (U/ml)	0.46 \pm 0.03	0.36 \pm 0.03	0.71 \pm 0.09*	0.37 \pm 0.05++

Each group consists of eight rats. *: $p < 0.05$; **: $p < 0.001$; compared to control group. +: $p < 0.05$; ++: $p < 0.01$; +++: $p < 0.001$; compared to ISO group. RES: Resveratrol (10 mg/kg/i.p); ISO: Isoproterenol (5 mg/kg/i.p); APs: Acid phosphatase.

**FIGURE 2.** (A) Na^+/K^+ ATPase activity, (B) ATP levels in the heart tissue of the control, resveratrol (RES), isoproterenol (ISO), ISO-RES groups. Data are presented as means \pm SEM. For each group $n=8$.

** : $p < 0.01$; *** : $p < 0.001$ versus control group; + : $p < 0.05$; $p < 0.01$ versus ISO group.

Cardiac Na^+/K^+ ATPase activity and ATP content decreased in the ISO group compared to the control group ($p < 0.01$ – 0.001 ; Fig. 2A, B, respectively). These values indicating membrane damage and impaired transport function increased in the ISO-RES treated group and returned to the control group values ($p < 0.05$).

**FIGURE 3.** (A) The representative images of nitrocellulose membranes (B) Caveolin-3 levels in the heart tissue of the control, resveratrol (RES), isoproterenol (ISO), ISO-RES groups. Data are presented as means \pm SEM. For each group $n=8$.

** : $p < 0.01$; *** : $p < 0.001$ versus control group; + : $p < 0.05$ versus ISO group.

ISO caused a significant reduction in the cav-3 level ($p < 0.001$, according to the control group). Although, in the ISO-RES group, cav-3 content of the tissue was preserved ($p < 0.01$), this was significantly different from that of the control group ($p < 0.001$; Fig. 3A, B).

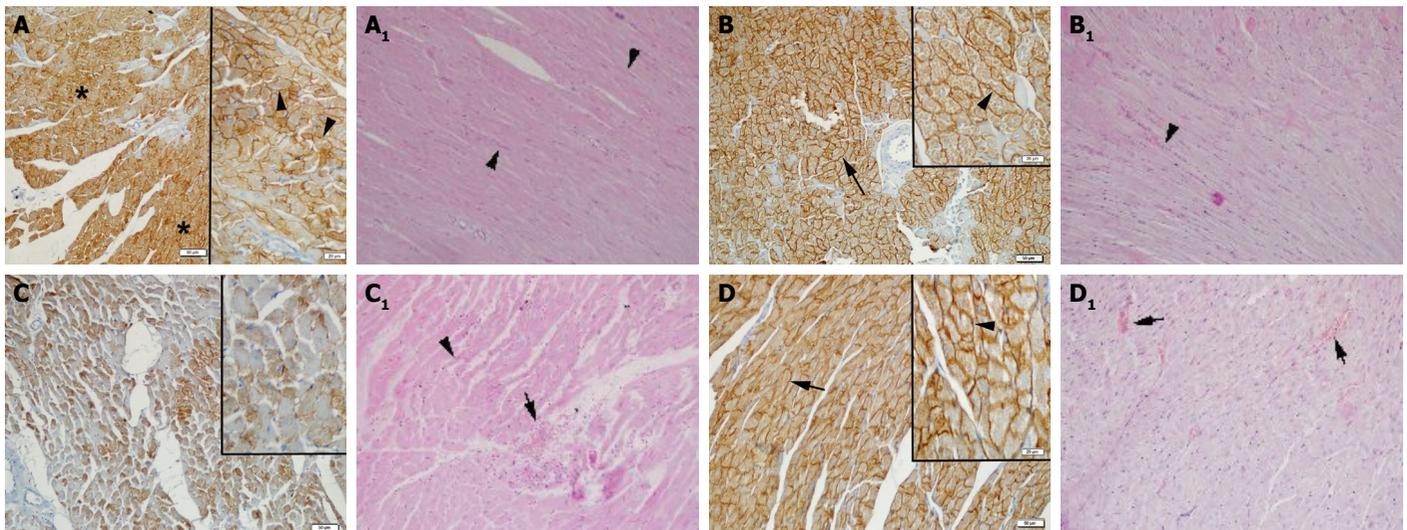


FIGURE 4. Caveolin-3 (CAV-3) immunostaining; **(A)** control group, strong staining of cardiac muscle cell membrane (arrowheads), regular muscle cell bundles (*), **(B)** RES group, strong staining pattern of membranes (arrowhead) similar to that of control group and regular muscle bundles (arrow), **(C)** ISO group, faint staining pattern of cellular membranes **(D)** ISO+RES group, the increased staining density of cav-3 was observed along with membranes (arrowhead) note the muscle bundles (arrow). Magnification X200, insets X 400. Hematoxylin-Eosin staining; **(A₁)** control group, normal morphology of both muscle bundles (arrowheads) and interstitium (*), **(B₁)** RES group, regular cardiac muscle morphology (arrowhead), **(C₁)** ISO group, irregular among muscle bundles (arrowhead) and prominent congestion (arrow), **(D₁)** ISO+RES group, regression of congestion (arrows), Magnification X200.

RES: Resveratrol (10 mg/kg/i.p); ISO: Isoproterenol (5 mg/kg/i.p).

Regarding the immunostaining of cav-3, both the control and RES (Fig. 4A, B, respectively) groups presented prominent cell membrane staining, whereas the membranes were faintly stained in the saline-treated ISO group (Fig. 4C). Similar to control and RES groups, the ISO-RES group presented strong staining of membranes (Fig. 4D). In both control (Fig. 4A₁) and RES (Fig. 4B₁) groups, the regular alignment of cardiac muscle cells was observed. In the ISO group, the cardiac muscle bundles were irregular and capillary obstruction was noteworthy (Fig. 4C₁). In the ISO-RES group, the layout of cardiac muscle bundles was observed better than the ISO group, and the capillary congestion was diminished (Fig 4D₁).

DISCUSSION

In the present study, the dietary flavonoid RES was investigated against ISO-induced myocardial damage. Previous studies have reported that ISO results in lipid peroxidation and cardiac dysfunction via the generation of ROS [18]. Quinine metabolites produced by ISO are also involved in the production of oxidative stress, because they increase superoxide and hydrogen peroxide radicals, and deplete endogenous antioxidants in rat my-

ocardium [19] MDA is increased when the generation of free radicals is increased and/or antioxidant defense is decreased [18]. In our study, ISO caused an elevation in cardiac MDA content, demonstrating oxidative damage, while RES markedly prevented this increase. Free radical scavenging antioxidants are the major cellular defenses against oxidative injury, and it has been documented that myocardial injury due to ISO is accompanied by depleted myocardial GSH levels [19, 20]. The decreased cardiac levels of this antioxidant following ISO administration confirm the consumption of the endogenous antioxidant. Furthermore, in the current study, we determined that GSH levels in ISO-injected rats were maintained when RES treatment was added. In our previous study conducted with another model of cardiotoxicity induced by doxorubicin, GSH level was increased with RES treatment [8]. In addition, Toklu et al. [9] reported that RES improves cardiovascular function both by oxidative damage that arose from hypertension and by increasing intracellular antioxidants. These findings suggested that RES exerts a blood pressure-independent cardioprotective effect, which may be due to its antioxidant activity.

Oxygen-free radicals generated during ischemia cause the further release of lysosomal enzymes, as well as damage directly to the myocardium [21]. Ravens and Gudbjarnason [22] have observed that lysosomal hydrolytic enzyme release is the major cause in the occurrence of ischemia-induced myocardial injury. Lysosomal enzymes are responsible for the autophagic digestion in special areas of the cytoplasm [23]. According to our results, we determined that the lysosomal enzyme activities indicating the level of myocardial injury were increased in the ISO group. In myocardial injury, activated inflammatory cells in the heart. MPO activity plays an important and central role in the generation of oxidants by neutrophils [24]. As the neutrophils are activated, MPO is released, which causes oxidation of proteins, carbohydrates, lipids, and nucleic acids [25]. Chemotactic substances released from neutrophils further promote the migration of neutrophils to tissue and thereby increase the tissue damage [26]. In the current study, RES suppressed the MPO activity, implicating that RES limits the infiltration of neutrophils into the injured myocardium. The results in our study are consistent with the findings of Sumitra et al. [27], who found that increased activity of MPO has an essential role in ISO-induced myocardial tissue damage. Our data parallel to the hypothesis 'inflammatory cells cause fibrosis to progress' suggests that, as assessed by elevated cardiac collagen content, the increase in ISO-induced fibrotic activity is reduced by RES treatment, and RES has an inhibitory effect against inflammation.

It was demonstrated that ISO caused the depletion of myocardial ATP levels, while antioxidant treatment significantly restored this nucleotide [28]. Similarly, in our study, cardiac ATP levels of ISO-induced rats were decreased, while RES treatment effectively prevented these reductions. Recently, it was shown that RES increased cardiac ATP and improved ventricular function [29], which is known as a major target of RES. In the pathogenesis of ischemic injury, impaired distribution of ions across the membrane through ionic pumps is postulated to be a major causative factor [30], while heart failure following ischemic heart disease is characterized by reduced myocardial Na^+/K^+ ATPase [31]. In the current study, we showed the decreased activity of Na^+/K^+ ATPase in the tissue of ISO-induced rats. Jayachandran et al. [32] showed that Na^+/K^+ ATPase was inhibited in ISO-induced myocardial infarction, while they also demonstrated that antioxidant therapy and membrane-stabilizing potential increased the enzyme activity and protected against ISO-induced cardiotoxic damage. In

our study, the activity of Na^+/K^+ ATPase was increased in ISO-induced rats upon administration of RES, which may have maintained membrane integrity and thereby protected the myocardium from excessive injury.

Cav-3 is a structural component of muscle caveolae, which are situated on the cytoplasmic surfaces of the sarcolemmal membrane, contributing to the stabilization of plasma membrane integrity, regulation of ion channels, vesicular trafficking and signal transduction [33]. In the current study, ISO-induced myocardial infarction downregulated cav-3 expression. In support of our findings, it was shown that beta-adrenergic receptor stimulation has also downregulated cav-3 in both cardiac muscles [34]. The current findings demonstrate that RES treatment significantly increased the expression of cav-3 in ISO-induced rats. Thus, these results suggest that the regulation of cav-3 status might be a critical mechanism in the protective action of RES against ISO-induced myocardial infarction.

Conclusion

Our results showed that increased lipid peroxidation, MPO activity and collagen content in the cardiac tissue along with the significant reductions in GSH, ATP content and Na^+/K^+ ATPase activity is due to the oxidant effects of the catecholamine ISO. Furthermore, ISO caused significant elevations in serum levels of LDH and CK, activities of lysosomal enzymes with a concomitant reduction in caveolin-3 levels. On the other hand, RES treatment reversed the oxidative stress, prevented the severity of ISO-induced myocardial injury, and improved cardiac dysfunction as verified by echocardiographic measurements. In the light of above-mentioned data, RES can be assumed as a promising agent in ameliorating the oxidative injury of the myocardium.

Ethics Committee Approval: Approval was obtained from the Marmara University Animal Care and Use Committee (43.2007.mar.) for all experimental protocols.

Conflict of Interest: No conflict of interest was declared by the authors.

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Authorship Contributions: Concept – AOS, OY, GS; Design – AOS, AA, ST, SC, MA; Supervision – BCY, GS; Fundings – MT, AA, NO, ZK; Methodology – AOS, AA, OY, ST, SC, NO, MA; Materials – AOS, AA, OY, ST, SC, NO, MA; Data curation – AOS, ZK, MT, AA; Validation – AOS, AA, BCY, GS; Formal analysis – AOS, BCY, GS; Software – AOS, AA, BCY, GS; Literature search – AOS, AA, ST, OY; Writing – AOS, AA, ST, OY, MT; Review and editing – AOS, AA, ST, OY, SC, NO, MA, ZK, BCY, MT, GS.

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