

Protective Effects of Resveratrol in Ischemia-Reperfusion Injury of Skeletal Muscle: A Clinically Relevant Animal Model for Lower Extremity Ischemia

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Abstract

Ischemia and reperfusion injury of the skeletal muscle is a common and serious condition observed in patients admitting to peripheral vascular surgery, interventional radiology and cardiology departments. Resveratrol (RVT) being a strong natural antioxidant is found in deal of red wine and Mediterranean diet. In the present study, male Sprague-Dawley rats were randomized into two groups of equal size. The first group was the control group, and these rats were administered with tap water with a gastric tube for fourteen consecutive days once daily. According to the same protocol, the rats in the second group were treated with tap water containing 20 mg/kg RVT. All the rats in the two groups were subjected to acute hind limb ischemia through clamping of the abdominal aorta for 120 min. Following this procedure, 60 minutes of reperfusion was applied by reestablishing blood flow in both iliac arteries. Ischemic damage in the skeletal muscle tissue was assessed by measuring myoglobin, lactate dehydrogenase, creatinine phosphokinase, aspartate transaminase enzymes in venous blood samples obtained at the end of the reperfusion period. Oxidative stress caused by reperfusion was determined by measuring MDA, carbonyl and protein sulphhydryl levels in quadriceps muscle tissue retrieved at the end of the experiment. In Group II rats, all the measured ischemic enzymes and the markers of oxidative stress reflected robust anti-ischemic properties obtained by RVT administration. The data from both groups revealed statistically significant protection against acute skeletal muscle ischemia and reperfusion injury in Group II rats, compared to Group I. As a major dietary flavonoid RVT can protect the skeletal muscle tissue against global ischemia and reperfusion injury because of its strong antioxidant and cytoprotective properties.

Key Words: resveratrol, antioxidant, ischemia reperfusion injury, protection, skeletal muscle, lower extremity, hind limb ischemia.

Introduction

Skeletal muscle ischemia and reperfusion injury

of the extremities, local and systemic effects, thereof are serious clinical problems in patients admitted to peripheral vascular surgery, interventional radiology

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and cardiology departments. Together with re-initiation of blood flow following ischemia, reperfusion injury is induced in the tissue; by triggering inflammatory reactions (9), and through deleterious effects of free oxygen radicals (FOR) generated by reoxygenation (27), the tissue is further damaged, compared to the damage induced only by ischemia (8).

Epidemiological studies conducted in Southern France demonstrated that ischemic heart disease rates were lower in this region of the country compared to others, despite significant smoking and fat-rich dietary habits (16). This phenomenon called French paradox was found to be due to the consumption of significant quantities of red wine (21). Resveratrol (RVT) (3,4', 5-trihydroxystilbene) is a strong natural antioxidant of polyphenolic structure found in grapes and red wine and has been indicated as the agent responsible for the French paradox in the studies performed (14). Several clinical and experimental studies have demonstrated that RVT inhibited platelet aggregation (1), and that due to lipid peroxidation, it prevented apoptosis (7). It also plays a protective role against coronary artery disease (18). In I/R models developed in different studies, RVT was shown to protect cerebral tissue (10) and cardiac muscle (11) from tissue damage caused by oxidative stress which was created during reperfusion. Its strong antioxidant properties were emphasized.

In the literature, ischemia/reperfusion studies with RVT have focused on cardiac tissue in particular. However, its strong antioxidant properties have not been investigated in ischemia and reperfusion of the skeletal muscle frequently encountered in clinical practice. This study aims at investigating the protective effects of RVT in skeletal muscle injury of rats induced by the acute ischemia and reperfusion of the lower extremities.

Materials and Methods

Animals

This study was performed at the School of Medicine, Osmangazi University after obtaining the approval of the Medical Ethics Committee. Male Sprague-Dawley rats weighing 250-300 were used. All the animals were fed with a standard ration (Oguzlar Yem, Eskişehir, Turkey) and allowed to drink tap water. In the environment where their cages were kept, there were 12 h cycles of light and darkness, and the room temperature was kept at 20- 25°C. All the phases of the experiment were carried out under sterilized conditions.

Drug

Resveratrol (Sigma, St. Louis, MO, USA) was

dissolved in ethanol and diluted in tap water. 600 mg/l concentration solutions were prepared for oral administration. The final concentration of ethanol in the prepared cocktail was 0.2% (6).

Study Protocol

Thirty adult Sprague-Dawley rats were randomized equally into two groups.

Group 1 (n = 15): Control group. Before the study, these animals were administered with tap water containing 0.2% ethanol through a gastric tube for fourteen days once daily.

Group 2 (n = 15): Experimental group. Before the study, these animals were administered with tap water containing RVT at a dose of 20 mg/kg through a gastric tube once per day for fourteen days.

Since chronic RVT and ethanol exposure didn't lead to any deleterious effects in the rats, the rats were used for this study 24 h following this treatment. These rats were anti-coagulated with 300 IU/kg intra-peritoneal heparin before the study. Ten min later, 50 mg/kg sodium pentothal was administered intra-peritoneally for anesthesia. Under anesthesia, abdomen was cleansed with betadine (10% povidone iodine solution) and abdominal cavity was opened with a median mini incision. Abdominal aorta and bilateral iliac arteries were explored. Abdominal aorta was occluded 1cm above the iliac bifurcation with micro-vascular clamp and ischemia was applied to both lower extremities for 120 min. At the end of 120 min the clamp was removed, and 60 min of reperfusion was applied by reestablishing blood flow in both iliac arteries. Throughout all these procedures, body temperature of the rats was monitored with a rectal probe and was kept stable at 37°C with the help of a heated surgical table. The rats were sacrificed at the end of the procedure.

Biochemical Assays

Ischemic Enzymes: At the end of the study, to identify the ischemic damage occurring in the skeletal muscle tissue, myoglobin, creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), and aspartate transaminase (AST) enzymes were measured. A catheter was placed to the right femoral vein at the 60th min of the reperfusion and venous blood was obtained. These samples were centrifuged at 3000 rpm for 10 min. Separated serum samples were transferred to different tubes and stored at -80°C for further analysis.

Markers of Oxidative Stress: To measure the oxidative stress caused by reperfusion injury and to compare it among the groups, MDA, carbonyl, and protein sulphydryl levels were measured in quadriceps

muscle tissue. While collecting blood samples from right femoral vein catheter, left iliac artery was simultaneously occluded and left quadriceps femoris muscle was rapidly removed with its sheath and stored at -80°C . At the end of the study, all the prepared tissues were homogenized with Ultra Turrax homogenizer (IKA T18 basic, Wilmington, NC, USA) in 0.1M phosphate buffer (pH 7.4). Homogenates were centrifuged at 5000 rpm at $+4^{\circ}\text{C}$ for 10 min. The obtained supernatants were centrifuged and the end-product of lipid peroxidation MDA was measured with thiobarbituric acid method (19). Following the reaction of supernatants with dinitrophenylhydrazine, amount of protein carbonyl was measured by colorimetric technique and the degree of protein oxidation was demonstrated (17). For measuring the level of sulphhydryl, method of Butler *et al.* (15) was employed.

Analysis of Data

All the biochemical measurements obtained from Group I and Group II were analyzed with Student's *t* test. Results were expressed as mean value \pm standard error (SEM). *P* value < 0.05 was accepted as being statistically significant.

Results

Ischemic Enzymes

Myoglobin: The myoglobin measurements of the blood samples obtained after reperfusion were significantly higher in control group than in RVT group ($P = 0.041$) (Fig. 1-A).

CPK: CPK levels of Group II were significantly lower than those of Group I ($P = 0.035$) (Fig. 1-B).

LDH: LDH levels obtained from Group II were significantly lower than levels in Group I ($P = 0.026$) (Fig. 1-C).

AST: AST measurements of Group II were significantly lower than the measurements in Group I ($P = 0.031$) (Fig. 1-D).

Markers of Oxidative Stress

MDA: MDA levels measured in the supernatants obtained from the left thigh of the rats after the experiment were higher in Group I than in Group II. The difference between the two groups was statistically significant ($P = 0.033$) (Fig. 2-A).

Carbonyl: Carbonyl levels of Group I were higher than that of Group II. The difference between the groups was statistically significant ($P = 0.042$) (Fig. 2-B).

Protein Sulphydryl: The sulphydryl levels

obtained from Group II were significantly higher than the levels in Group I ($P = 0.039$) (Fig. 2-C).

Discussion

In this study, natural antioxidant substance RVT was demonstrated to have protective effects on acute ischemia and reperfusion injury of the skeletal muscle in lower extremities. Clinical presentation of I/R seen in lower extremities is frequently encountered in vascular surgery departments due to causes like acute arterial occlusions and peripheral vascular interventions. In such patients, in addition to time-dependent damage caused by ischemia; oxygen and FOR like hydrogen peroxide, nitric oxide and peroxynitrate generated during reperfusion all contribute to the tissue injury by triggering oxidative stress (13). Under physiological conditions, intrinsic antioxidant enzyme systems like SOD, glutathione peroxidase and catalase limit tissue concentrations of FOR produced by cellular aerobic reactions (13). However, in chronic disease states like diabetes mellitus, heart failure or ischemia, the amount of FOR and their toxic effects go beyond the scavenging capabilities of antioxidant enzyme systems and result in oxidative stress (13). Especially during the early stages of reperfusion, both due to the inadequacy of antioxidant enzyme systems and to excessive production, FOR levels increase tremendously resulting in the strongest phase of the reperfusion injury in the tissues involved (30). In line with this mechanism, FOR scavenging molecules and substances with antioxidant properties support the cellular antioxidant systems in an attempt to limit or even eliminate reperfusion injury (4).

RVT as announced by French paradox is a strong natural antioxidant of polyphenol structure. The performed studies have demonstrated that its antioxidant efficacy was far better than that of vitamin E (7). The efficacy of antioxidant agents are directly related to their tissue concentrations reached during oxidative stress (22). RVT administration at the dose of 20 mg/kg once a day was previously demonstrated as the effective dosage for protection against oxidative stress (26). In our study as well, rats were administered with RVT at a dose of 20 mg/kg once a day for 14 days.

Enzymatic parameters (CPK, myoglobin, AST, LDH) measured for demonstrating ischemia induced muscle injury were lower in the group receiving RVT. Under the light of the performed studies, as well as in tissues sensitive to ischemia such as heart and kidneys preconditioning is very well known to be protective for I/R injury in the skeletal muscle too (24). Other than its antioxidant effects, RVT increases the secretion of adenosine and NO; most important mediators of preconditioning and RVT thereby demonstrates a pharmacological preconditioning effect in these

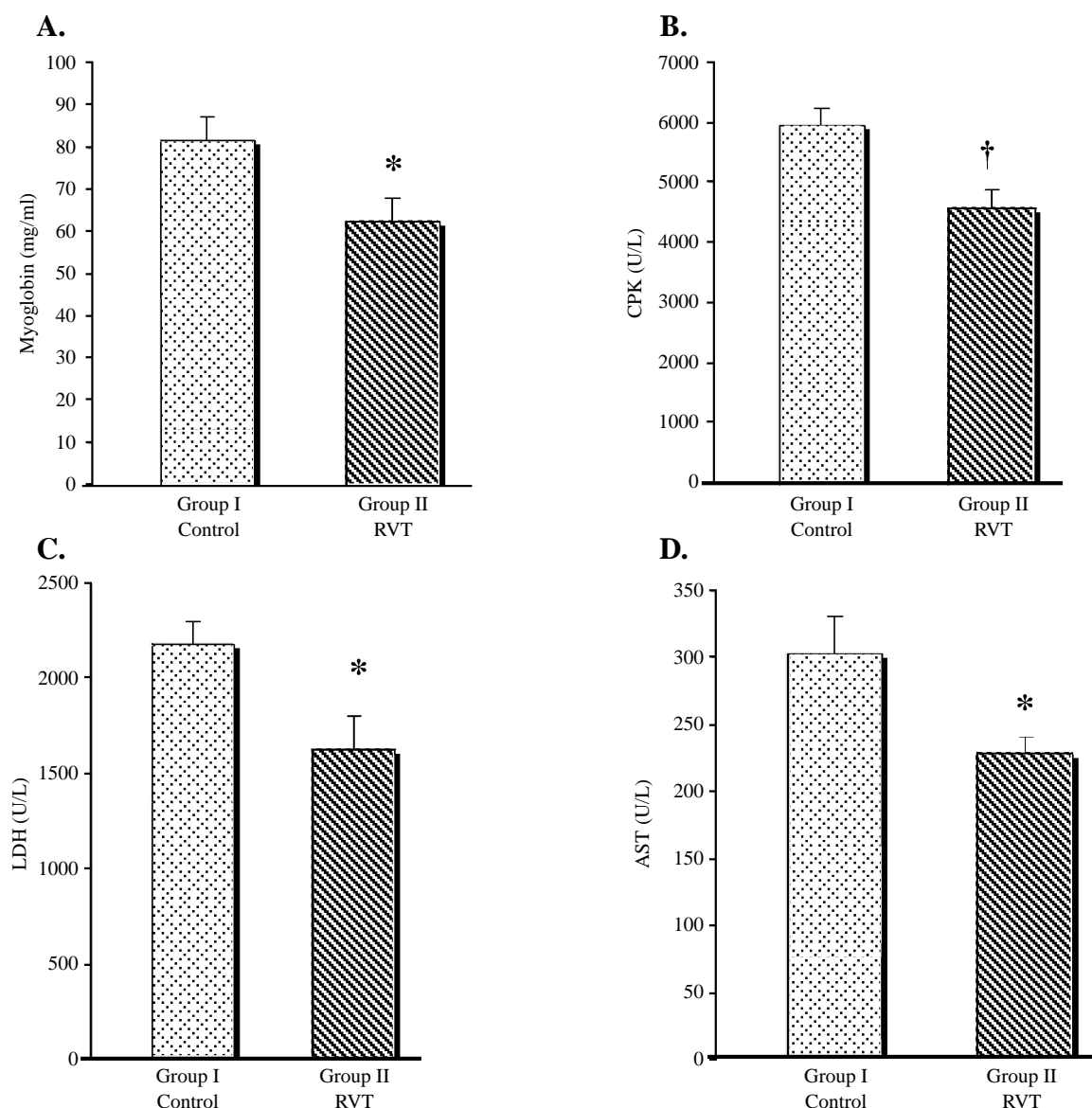


Fig. 1. Effects of RVT on myoglobin (panel A) and creatinine phosphokinase (panel B), lactate dehydrogenase (panel C), aspartate transaminase (panel D) levels. Results are presented as the mean \pm SEM. * $P < 0.05$ and † $P < 0.01$.

tissues (2, 20). Enzymes dictating ischemia induced muscle injury were lower in rats receiving RVT, which can be explained by the protective features of the preconditioning like effects of RVT molecule.

In an experimental model, RVT administration resulted in four-fold increased levels of plasma NO in rats compared to the control group (12). The primary source of these high levels of serum NO was the vascular endothelium (5). Today, we very well know that through regulation of cGMP; NO decreases the consumption of oxygen in the tissues and increases the resistance to ischemia (29). Furthermore, *via* its effects on vascular smooth muscle it results in a strong vasodilator response. A study on isolated rat aorta has demonstrated that RVT created a powerful

vasodilator effect by increasing the concentrations of NO (3). Together with the available data, we believe that in animals administered with RVT, the increased tissue concentrations of NO result in vasodilatation and increased blood flow; the accompanying decrease in the consumption of oxygen further contributes to the protection of skeletal muscle from ischemia.

During reperfusion induced oxidative stress, due to its barrier function cell membrane is the first region to be affected. Generation of excessive amounts of FOR, trigger lipid peroxidation and result in the destruction of polyunsaturated fatty acids found in the composition of cell membrane (12). Among lipid peroxidation products, peroxy radicals were identified to have important roles in cellular injury (28). With

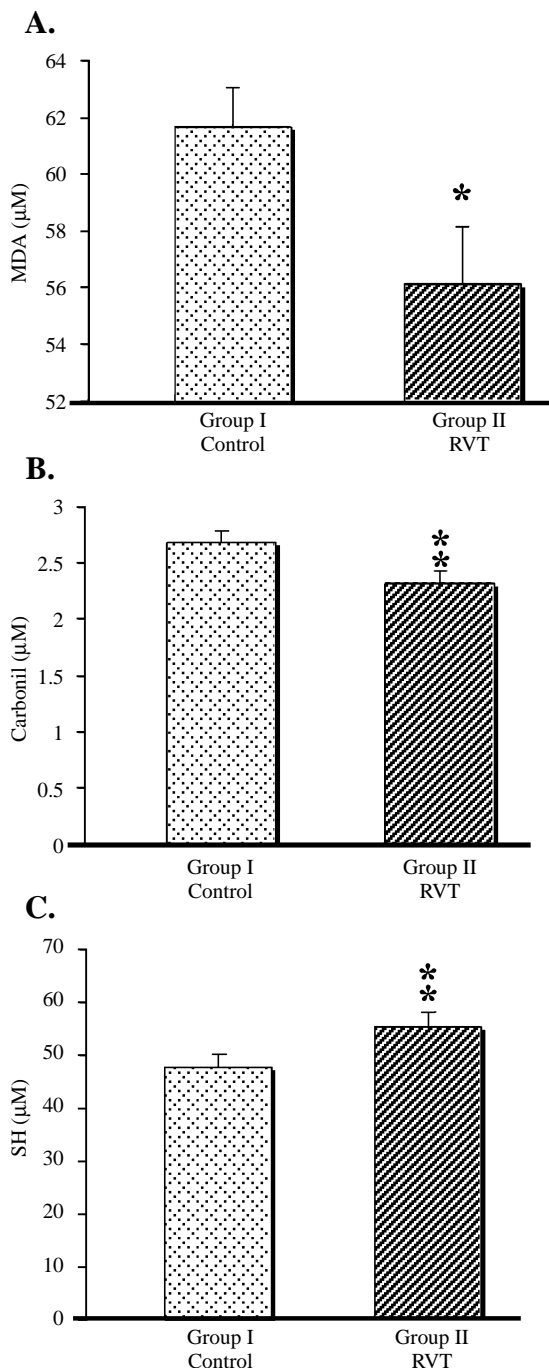


Fig. 2. Effects of RVT on oxidative stress indicators, MDA (panel A), carbonil (panel B) and SH (panel C). Results are expressed as mean \pm SEM. * $P < 0.05$.

the continuation of oxidative stress; MDA is generated by lipid peroxidation reactions (25). In the studies performed, RVT was shown to prevent lipid peroxidation (7), and shown to decrease the deleterious effects by scavenging peroxyl radicals (28). In the present study, the finding of low levels of MDA in the group receiving RVT compared to the control group supports the hypothesis that RVT shows oxidative stress reducing

effects by scavenging peroxyl radicals in skeletal muscle tissue. Similarly, levels of carbonyl, the product of protein oxidation, were also lower in the group receiving RVT indicating that oxidative stress was of lesser degree in the rats of this group (17). Because of their very short half lives, FOR responsible from oxidative injury cannot be directly measured. Thus, it is more versatile to use measurements of cellular antioxidant molecules (GSH, SH) for demonstrating the degree of oxidative damage (23). SH levels measured in our study were higher in Group II rats, showing that with its strong antioxidant properties RVT supports the tissue during oxidative stress while limiting oxidative injury. In conclusion, this study demonstrates us that RVT is a strong natural antioxidant of polyphenol nature protecting skeletal muscle tissue against deleterious effects of ischemia. Because of its strong antioxidant properties, oral administration of RVT for 14 days, diminishes the severity of oxidative stress generated by reperfusion ensuing ischemia and plays a protective role for skeletal muscle cells against reperfusion injury.

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