

The Effect of Picroside-2 on Erythrocyte Deformability and Lipid Peroxidation in Streptozotocin-Induced Diabetic Rats Subjected to Left Anterior Descending Artery-Ischaemia Reperfusion

Streptozosin ile Diyabet Oluşturulan Sıçanlarda Sol Ön İnen Arter-İskemi Reperfüzyonunda Pikrozid-2'nin Eritrosit Deformabilitesi ve Lipid Peroksidasyonu Üzerine Etkisi

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ABSTRACT

Aim: Diabetes mellitus (DM) is a chronic metabolic disorder principally characterized by an elevation in oxidative stress levels. Ischaemia-reperfusion (IR) injury starts a cascade of events that lead to tissue ischaemia and cellular damage produced by reperfusion causing an inflammatory like response. Erythrocyte deformability and plasma viscosity are important clinical implications for organ and tissue perfusion. Recent studies have found that picroside-2 has antioxidant, neuroprotective and anti-inflammatory effects. The aim of our study was to investigate the effects of picroside-2 on erythrocyte deformability and lipid peroxidation in streptozotocin-induced diabetic rats subjected to left anterior descending (LAD) artery IR.

Methods: The animals were randomly assigned to one of five experimental groups. In Group (control) C, DC (diabetes-control group), and DP (diabetes-picroside-2 group) neither coronary artery occlusion nor reperfusion were performed in the control rats. In Group DIR, a branch of the LAD artery was occluded for 45 minutes followed by 90 minutes of reperfusion to produce IR. In Group DIRP, picroside-2 was administered via 10 mg.kg⁻¹ intraperitoneal (IP) route 30 minutes before ligating the LAD artery. Serum malondialdehyde and nitric oxide activities were investigated to document lipid peroxidation and erythrocyte deformability index.

Results: Deformability index was notably increased in diabetic rats (p<0.0001). It was notably increased in Group DIR when compared to Group C, DC, DP and DIRP (p<0.0001, p=0.009, p=0.013, p=0.009, respectively). MDA level and NO activity were also higher in IR group than the other groups.

Conclusion: Erythrocyte deformability index was decreased in rats with diabetes and IR injury. This injury may lead to further microcirculatory problems. Picroside 2 was shown to be useful in reducing the side effects of this kind of injury.

Key Words: Erythrocyte deformability, myocardial ischaemia reperfusion, experimental diabetes, picroside-2, MDA, NO

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ÖZET

Amaç: Diyabetes mellitus (DM), özellikle oksidatif stres seviyelerinde yükselme ile karakterize edilen kronik bir metabolik bozukluktur. İskemi-reperfüzyon (İR) hasarı, doku iskemisi ve reperfüzyonla üretilen hücresel hasara yol açan, bir dizi inflamatuvar cevap benzeri olay sonucunda başlar. Eritrosit deformabilitesi ve plazma viskozitesi, organ ve doku perfüzyonu için önemli klinik belirteçlerdir. Son yıllardaki araştırmalar, pikrozid-2'nin antioksidan, nöroprotektif ve anti-inflamatuvar etkilere sahip olduğunu bulmuştur. Çalışmamızın amacı, streptozosin ile diyabet oluşturulan, sol ön inen (LAD) arter için İR uygulanan ratlarda pikrozid-2'nin eritrosit deformabilitesi ve lipid peroksidasyonu üzerine etkilerini araştırmaktır.

Yöntem: Hayvanlar rasgele beş gruba ayrıldı. Grup K, DK (diyabet kontrol grubu) ve DP (diyabet-pikrozid-2 grubu) ratlarında koroner arter oklüzyonu veya reperfüzyon uygulanmadı. Grup DİR'de LAD arterinin bir dalı 45 dakika boyunca kapatıldı, bunu takiben İR hasarına neden olmak için 90 dakika reperfüzyon uygulandı. Grup DİRP'de LAD arterinin bağlanması 30 dakika önce 10 mg.kg⁻¹ intraperitoneal (IP) yolu ile pikrozid-2 uygulandı. Serum malondialdehit ve nitrik oksit aktiviteleri, lipid peroksidasyonu ve eritrosit deformabilite indeksi değerlendirildi.

Bulgular: Deformabilite indeksi diyabetik ratlarda belirgin şekilde arttı (p<0.0001). Grup DİR'de Grup K, DK, DP ve DİRP'ye kıyasla belirgin şekilde arttı (p<0.0001, p=0.009, p=0.013, p=0.009, sırasıyla). MDA düzeyi ve NO aktivitesi İR grubunda diğer gruplardan daha yüksekti.

Sonuç: Diyabet ve İR hasarı olan ratlarda eritrosit deformabilite indeksi azaldı. Bu hasar, daha ileri mikrosirkülasyon sorunlarına neden olabilir. Pikrozid-2'nin bu tür hasarlanmanın yan etkilerini azaltmada yararlı olduğu gösterildi.

Anahtar Sözcükler: Eritrosit deformabilitesi, miyokardiyal iskemisi reperfüzyonu, deneysel diyabet, pikrozid-2, MDA, NO

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INTRODUCTION

In last two or three decades the prevalence of diabetes mellitus (DM) has increased exponentially throughout the world, and experts estimate that it will increase by 200% in the next several decades (1–4). Diabetes mellitus (DM) is an increasingly common medical condition. Diabetes mellitus is an increasingly common disease that affects people of all ages. Diabetic patients require perioperative care more frequently and physicians need to pay extra attention to this. Premature mortality, microvascular and cardiovascular complication risk is significantly increased in patients with diabetes. These patients tend to be sicker than most non-diabetic patients; so they place a proportionally larger burden on anaesthetic services (2, 5, 6). Thus, more careful perioperative care is necessary for these patients (7).

Diabetes has been associated with a 50% increased early mortality following coronary artery bypass graft surgery (5). Likewise, the Framingham Heart Study reported higher incidences of painless myocardial infarction in patients with diabetes than without (39% vs 22%) (8). Cardiac surgery with cardiopulmonary bypass is inevitably associated with a systemic inflammatory response and ischaemia-reperfusion (IR) injury affecting multiple organs (1, 2).

Highly reactive molecules called free radicals can cause tissue damage by reacting with polyunsaturated fatty acids in cellular membranes. Circulating red blood cells (RBC) are particularly more susceptible to oxidative stress. During IR, induced oxidative stress can result in the oxidation of lipids and can cause significant modification in RBC shape or in cell membrane with the consequent alteration of cellular deformability. Erythrocyte deformability is crucial for the maintenance of normal circulation. Optimal deformability facilitates the passage of RBC through narrow capillaries in the microcirculation and reduces blood viscosity at high shear rates in large blood vessels (9).

In vitro and in vivo studies indicate that lipid peroxidation associated with diabetic complications and that this plays a critical role in the pathogenesis of diabetic complications. So control of lipid peroxidation is very important. A variety of endogenous and exogenous antioxidant mechanisms serve to control this peroxidative process (10-15).

Normally, endothelial NOS synthesizes low levels of nitric oxide (NO) and it regulates vascular tone. During IR injury, the NO production increases and toxic effects of NO become prominent (16). The hemorheological parameters in DM are often disturbed. These parameters include (but are not limited to) hematocrit, plasma proteins, erythrocyte aggregation, and erythrocyte deformability (17).

Picrorhiza scrophulariiflora belongs to the plant family, *Scrophulariaceae*. The roots of this plant are of benefit to health and often used in traditional Chinese medicine to treat a number of conditions (18). Extracts of the roots contain various terpenoids and glycosides and picroside-2 is one of the main active constituents of the extracts. Numerous published studies have shown that picroside-2 has a wide range of pharmacological effects, including neuroprotective, hepatoprotective, anti-apoptosis, anti-cholestatic, anti-inflammatory and immune-modulating activities (19–21). Picroside-2 has also been shown to have a protective effect against IR in other organs, including the brain (22), renal (23), because of its anti-oxidative, anti-inflammatory and anti-apoptotic properties.

This study aims at investigating deformability changes and picroside-2's preventive role against these changes in lipid peroxidation and erythrocytes of diabetic rats during an experimental model of myocardial IR injury.

MATERIALS and METHODS

Animals and Experimental Protocol

This study was conducted in the Laboratory Animals Raising and Experimental Researches Center (GUDAM) Laboratory of Gazi University with the consent of the Experimental Animals Ethics Committee of Gazi University. All of the procedures were performed according to the accepted standards of the Guide for the Care and Use of Laboratory Animals.

In the study, 30 male Wistar Albino rats weighing between 230 and 290 g, raised under the same environmental conditions, were used. The rats were kept at 20-21°C in cycles of 12 hours of daylight and 12 hours of darkness and had free access to food until two hours before the anaesthetic procedure. The animals were randomly separated into five groups, each containing six rats.

Diabetes was induced by a single IP injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA), at a dose of 55 mg.kg⁻¹ body weight. The levels of blood glucose were measured for 72 hours following this injection. Rats were classified as diabetic if their fasting blood glucose (FBG) levels were over 250 mg.dL⁻¹, and only animals with FBGs of > 250 mg.dL⁻¹ were included in the diabetic groups (diabetes only, diabetes picroside-2, diabetes plus IR and diabetes plus picroside-2-IR). The rats were kept alive for four weeks after streptozotocin injection to allow the development of chronic diabetes before they were exposed to IR [21]. The rats were weighed before the study.

Rats were anesthetized with an IP injection of 100 mg.kg⁻¹ of ketamine. The chest was shaved with a small animal clipper. The trachea was cannulated and artificial respiration applied. Each animal was fixed in a supine position on the operating table. The chest was opened by left thoracotomy at approximately 2mm to the left of the sternum and followed by sectioning the fourth and fifth ribs. Rats were then immediately subjected to positive pressure artificial respiration with room air using a volume of 1.5 mL/100 g body weight at a rate of 60 strokes/min. Sodium heparin (500 IU.kg⁻¹) was injected via peripheral vein in the tail.

After incising pericardium, the heart was exteriorized with gentle pressure on the right side of the rib cage. An 8/0 silk suture attached to a 10-mm micropoint reverse-cutting needle was used to placed under the left main coronary artery. The heart was then carefully replaced in the chest and the animal was allowed to recover for 20 minutes.

There were five experimental groups. Group C (control; n=6), Group DC (diabetes-control; n=6), Group DP (diabetes-picroside-2), Group DIR (diabetes-IR; n=6) and Group DIRP (diabetes-IR-picroside-2; n=6) underwent left thoracotomy and received IP picroside-2 (Sigma Aldrich Co. Ltd. (CAS No: 39012-20-9, purity> 98%, molecular formula: C₂₃H₂₈O₁₃) administrated via 10 mg.kg⁻¹ IP route 30 minutes before ligating the LAD. A small plastic snare was threaded through the ligature and placed in contact with the heart. Applying tension to the ligature could then occlude the artery (45 minutes), and reperfusion was achieved by releasing the tension (90 minutes). However, after the above procedure, the coronary artery was not occluded or reperfused in the control, diabetic control and diabetic picroside-2 rats.

All the rats were given ketamine 100 mg.kg⁻¹ IP and intracardiac blood samples of rats were obtained. Erythrocyte packs were prepared using heparinized total blood samples. Deformability measurements were achieved by using erythrocyte suspensions with haematocrit level of 5% in phosphate buffered saline (PBS) buffer.

Deformability Measurements

Samples of blood were carefully taken and the measurement process was as fast as possible to avoid haemolysis of erythrocytes. The collected blood was centrifuged at 1000 rpm for 10 min. Serum and buffy coat on erythrocytes were removed. An isotonic PBS buffer was added to the collapsing erythrocytes and this was followed by centrifugation for 10 min at 1000 rpm. Liquid on the upper surface was removed. Finally, pure red cell packs were obtained from the washing process, which was repeated three times. Erythrocyte packs were mixed with the PBS buffer to generate a suspension with a value of 5% Hct. These erythrocyte suspensions were used for the measurement of deformability. Collection and deformability measurements of erythrocytes were performed at 22°C.

The constant-current filterometer system was used in the measurement of erythrocyte deformability. Samples to be measured were prepared with 10 mL of erythrocyte suspension and PBS buffer. The flow rate was maintained at 1.5 mL/min with an infusion pump. A 28 mm nucleopore polycarbonate filter with a 5 µm pore diameter was preferred. Pressure changes while the erythrocytes passed through the filter were detected by the pressure transducer, and the data was transferred to the computer with the help of an MP30 data equation system (Biopac Systems Inc, Commat, USA). The necessary calculations were performed with related computer programs by measuring the pressure changes at various times. Pressure calibration of the system was performed before each sample measurement. The buffer (P_r) and the erythrocytes (P_e) were passed through the filtration system and the changes in pressure were measured. The relative refractory period value (Rrel) was calculated by relating the pressure value of the erythrocyte suspension to the pressure value of buffer. Increasing Rrel in the deformability index was interpreted to adversely affect the erythrocytes' deformability.

Measurements of MDA levels and NO activities

Esterbauer method was applied in order to measure lipid peroxidation. Malondialdehyde reacted with thio-barbituric acid at 90-95 °C and resulted in pink chromogranin. After 15 min specimens rapidly cooled then absorbances were read at 532 nm spectrophotometrically. Results were represented as nmol.g^{-1} tissue protein (24). NO's stable oxidative metabolites' concentrations (NO_2^- ve NO_3^-) were measured in serum and NO production was determined. Measurement of nitrite concentration was performed with Griess reaction (25).

Statistical Analysis

The statistical analyses were performed with SPSS 20.0 software program and $p < 0.05$ was considered statistically significant. The findings were expressed as mean \pm standard error (SE). The data were evaluated with Kruskal-Wallis variance analysis. The variables with significance were evaluated with Bonferroni corrected Mann-Whitney U test.

RESULTS

Deformability index was significantly increased in diabetic rats ($p < 0.0001$), however it was similar in Group DC and DIRP ($p = 0.989$). It was significantly increased in Group DIR when compared to Group C, DC, DP and DIRP ($p < 0.0001$, $p = 0.009$, $p = 0.013$, $p = 0.009$, respectively) (Fig. 1). Relative resistance was increased in IR models.

MDA level was also higher in IR group than the other groups ($p = 0.001$, $p = 0.004$, $p = 0.003$, $p = 0.005$, respectively), (Figure 2). However, picroside-2 treatment in IR-P group resulted with similar MDA levels with the C, DC, and DP groups (Figure 2).

NO activity was also higher in IR group than the other groups ($p < 0.0001$, $p = 0.001$, $p = 0.030$, $p = 0.001$, respectively), (Figure 3). However, picroside-2 treatment in IR-P group resulted with similar NO activity with the DC, DP groups (Figure 3).

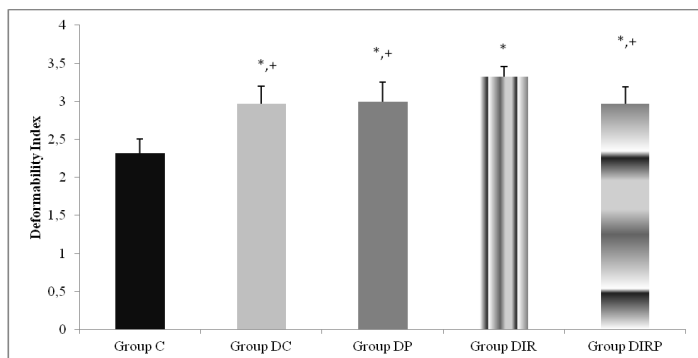


Figure 1: Erythrocyte deformability values of the groups. Each bar represents the mean \pm SE.

* $p < 0.05$ compared to Group C; + $p < 0.05$ compared to Group DIR

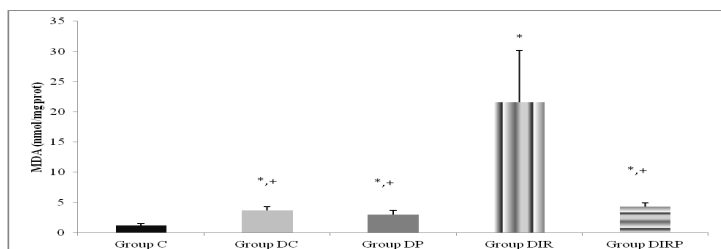


Figure 2: Malondialdehyde levels values of the groups. Each bar represents the mean \pm SE.

* $p < 0.05$ compared to Group C; + $p < 0.05$ compared to Group DIR

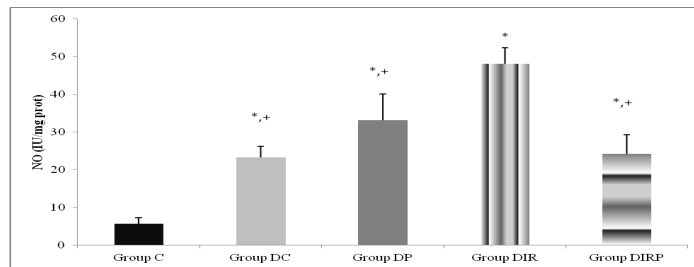


Figure 3: Nitric Oxide activity values of the groups. Each bar represents the mean \pm SE.

* $p < 0.05$ compared to Group C; + $p < 0.05$ compared to Group DIR

DISCUSSION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes mellitus has a high social and economic importance, as the number of diabetes patients continues to grow at an unprecedented rate throughout the world (26).

Lipid peroxidation causes loss of viscosity in biological membranes, decrease in membrane potential and cell injury due to increased permeability of hydrogen and other ions. One of the most important type of injury caused by free radicals is lipid peroxidation and it is measured by MDA production. It is one of the most common and well-known methods for evaluating IR injury (27).

Functional studies of the endothelium showed that hypoxia causes endothelial cell injury. Significant increase in endothelial NO release was observed after 150 seconds following re-establishment of arterial flow. NO inhibits degranulation by directly effecting GMP-cylase enzyme of the platelets and their aggregation (28).

Hemorheological parameters in DM are often disturbed. These parameters include (but are not limited to) hematocrit, plasma proteins, erythrocyte aggregation, and erythrocyte deformability (29). For migration of oxygen and vital molecules to the final organ capillaries and clearance of metabolic wastes, erythrocytes must be able to extend and curve and have the capability to move in these areas. This capacity, called "deformability", becomes more important in microcirculation. Altered erythrocyte deformability changes the erythrocytes' capacity of oxygen delivery and also helps the survival of the circulating erythrocytes (30-32).

In addition, the impaired perfusion at the tissue level observed as a complication of DM has been suggested to be primarily due to reduced erythrocyte deformability (33, 34). Additionally, metabolic changes and tissue perfusion due to cardiovascular problems may lead to inadequate recovery in plasma viscosity (35).

Previous studies showed various pharmacologic effects of picroside-2 such as antioxidant, antiapoptotic and antiinflammatory ones (19-23).

Cho et al. (17) demonstrated that blood viscosity was significantly high in diabetes. These results indicate that the consequent glucose elevation in blood plasma mainly affects RBCs and the vascular endothelial cells, including the capillary walls. The impaired tolerance of glucose or uncontrolled levels of blood glucose often causes microvascular complications in diabetes. Moreover, the impairment of erythrocyte deformability is attributed to the specific changes in the membrane structure. The oxidative stress due to high glucose concentrations causes damage to the erythrocyte membrane proteins, even with a relatively short exposure time (36).

Barnes et al. (37) reported that erythrocyte deformability was lower in the 14 diabetes patients with the most extensive micro-angiopathy than in the controls or the 22 diabetes patients with slight or no complications. They suggested that hyperviscosity and reduced erythrocyte deformability may be important and potentially treatable factors in the aetiology or progression of microcirculatory disease in diabetes. Similar to these previous studies, we also found that erythrocyte deformability was decreased in diabetes induced rats.

To the best of our knowledge, ours is the first study to show that IR of the diabetic rat heart results in significant negative changes that can be observed in erythrocyte deformability and that picroside-2 administration at the beginning of heart ischaemia can provide varying degrees of protection against the negative effects of variations in erythrocyte deformability.

As a conclusion, the results of this study clearly demonstrated that erythrocyte deformability is significantly altered in experimental myocardial IR injury in the diabetic rat. This might cause further problems in microcirculation. Thus, measurement of erythrocyte deformability might have a critical effect on the follow-up for IR injury. Additionally, picroside-2 administered before induction of ischaemia was observed to have protective effects on these alterations in myocardial IR injury. Other aspects of these findings, including clinical significance and practical applications, merit further experimental and clinical investigation.

Conflict of interest

No conflict of interest was declared by the authors.

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