

# Effect of picoside II on erythrocyte deformability and lipid peroxidation in rats subjected to hind limb ischemia reperfusion injury

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**Background:** Ischemia reperfusion injury (I/R) in hind limb is a frequent and important clinical phenomenon. Many structural and functional damages are observed in cells and tissues in these kinds of injuries. In this study, we aimed to evaluate the effect of picoside II on lipid peroxidation and erythrocyte deformability during I/R in rats.

**Methods:** Rats were randomly divided into four groups – each containing six animals (sham, I/R, sham + picoside II, and I/R + picoside II). The infrarenal section of the abdominal aorta was occluded with an atraumatic microvascular clamp in I/R groups. The clamp was removed after 120 minutes and reperfusion was provided for a further 120 minutes. Picoside II (10 mg·kg<sup>-1</sup>) was administered intraperitoneally to the animals in the appropriate groups (sham + picoside II, I/R + picoside II groups). All rats were euthanized by intraperitoneal administration of ketamine (100 mg·kg<sup>-1</sup>) and taking blood from the abdominal aorta. Erythrocytes were extracted from heparinized complete blood samples. Buffer (P<sub>T</sub>) and then erythrocytes (P<sub>E</sub>) were passed through the filtration system and the changes in pressure were measured to investigate the role of serum malondialdehyde and nitric oxide (NO) in lipid peroxidation and erythrocyte deformability index.

**Results:** Deformability index was significantly increased in the I/R group compared to groups sham, sham + picoside-II, and I/R + picoside-II ( $P < 0.0001$ ,  $P < 0.0001$ , and  $P = 0.007$ ). Malondialdehyde (MDA) and NO levels were evaluated. MDA level and NO activity were also higher in the I/R group than in the other groups. Picoside II treatment before hind limb I/R prevented these changes.

**Conclusion:** These results support that deformability of erythrocytes is decreased in I/R injury and picoside II plays a critical role to prevent these alterations. Further experimental and clinical studies are needed to evaluate and clarify the molecular mechanisms of action and clinical importance of these findings.

**Keywords:** hind limb ischemia reperfusion injury, lipid peroxidation, malondialdehyde, nitric oxide, erythrocyte deformability, picoside II

## Introduction

Ischemia reperfusion injury (I/R) in hind limb is a frequent and important clinical phenomenon. Reperfusion period following an ischemic insult may paradoxically cause increased rates of mortality and morbidity due to systemic complications. Local edema and muscle tissue necrosis are likely to be followed by systemic inflammatory response syndrome and multiple organ failure (kidney, respiratory, and circulatory system) as reperfusion advances.<sup>1-3</sup>

Swelling of cells, degeneration of cell skeleton structure, and loss of selective membrane permeability are characteristic features of reperfusion injury. These changes all end up with tissue edema and decreased capillary blood flow.<sup>4</sup>

Microcirculatory damage is the first target of I/R. Leukocyte–endothelial interactions cause transendothelial migration and tissue damage by triggering the release of reactive oxygen species and elastase.<sup>5</sup> Many tissues and cells such as erythrocytes may be damaged due to the exogenous sources of reactive oxygen species.<sup>6</sup> Deformability of erythrocytes is the key factor for the regulation of normal microvascular circulation. Viscoelastic properties of erythrocyte membrane are the major determinants of red blood cell deformability.<sup>7</sup>

Picoside II is one of the main active constituents isolated from *Picrorhiza scrophulariiflora*. The roots of this plant are of benefit and often used in traditional Chinese medicine for a number of conditions.<sup>8</sup> Picoside II has been shown to possess a wide range of pharmacological effects, including effects against oxidative stress and inflammation.<sup>9–11</sup> Picoside II has also been shown to protect against I/R in other organs, including the brain,<sup>12</sup> and kidneys,<sup>13</sup> due to its antioxidative, anti-inflammatory, and antiapoptotic properties.

Previous studies have shown that picoside II prevents the I/R-induced lipid peroxidation, but as far as we know this is the first report of picoside II's effect on lipid peroxidation and erythrocyte deformability in hind limb I/R injury. The primary purpose of this study was to investigate I/R injury-induced changes of hind limb in deformability of erythrocytes and the protective role of picoside II on increased lipid peroxidation and erythrocyte deformability alterations in experimental hind limb I/R injury.

## Materials and methods

### Animals and experimental protocol

This study was conducted in the GUDAM (Gazi Universitesi Laboratuvar Hayvanları Yetiştirme ve Deneysel Araştırmalar Merkez) Laboratory of Gazi University with the consent of the Experimental Animals Ethics Committee at Gazi University. All procedures were performed according to the accepted standards of the Guide for the Care and Use of Laboratory Animals.

In our study, 24 male Wistar Albino rats weighing between 175 and 230 g, raised under the same environmental conditions, were used. The rats were housed between 20°C–21°C with 12-hour daylight and 12-hour darkness cycles, and had free access to food until 2 hours before the anesthesia procedure. The animals were randomly separated

into four groups, each containing six rats. Midline laparotomy was done under ketamine (100 mg·kg<sup>-1</sup>) anesthesia.

Sham group: midline laparotomy was done alone without any additional surgical intervention. Blood samples were collected after 4 hours of follow-up and animals were sacrificed eventually.

Sham + picoside II group: midline laparotomy was done alone without any additional surgical intervention. Blood sample was collected after 4 hours of follow-up and animals were sacrificed eventually.

I/R group: midline laparotomy was done similarly. Infra-renal part of the abdominal aorta was clamped for 2 hours. After removing the clamp, reperfusion was established for another 2 hours. Finally, rats were sacrificed after collecting blood samples from their abdominal aorta.

I/R + picoside II group: Similar steps were followed but in addition to the procedure mentioned above, picoside II was supplied by Sigma-Aldrich Co. Ltd. (St Louis, MO, USA; CAS No: 39012-20-9, purity >98%, molecular formula: C<sub>23</sub>H<sub>28</sub>O<sub>13</sub>). The picoside II was diluted to a 1% solution with normal saline. The animals in the treatment group were administered an intraperitoneal injection of picoside II 30 minutes before ischemia (10 mg·kg<sup>-1</sup>); the animals in the control and I/R groups were injected with the same volume of 0.09% NaCl. Rats were sacrificed at the end of reperfusion period that lasted 2 hours, after collecting blood samples.

All the rats were given ketamine 100 mg·kg<sup>-1</sup> intraperitoneally and intraabdominal blood samples were taken. Erythrocyte packs were prepared using heparinized total blood samples. Measurements for deformability were performed using erythrocyte suspensions with 5% hematocrit in phosphate buffered saline (PBS).

### Deformability measurements

Samples of blood were taken very carefully and the measurement process was done as fast as possible to avoid hemolysis. Collected blood was centrifuged at 1,000×g for 10 minutes. Serum and buffy coat on erythrocytes were removed. Isotonic PBS buffer was added to collapsing erythrocytes and this mixture was centrifuged at 1,000×g for 10 minutes. 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 PBS buffer to generate a suspension with the value of 5% hematocrit. These erythrocyte suspensions were used for the measurement of deformability. Collection and deformability measurements of erythrocytes were done at 22°C.

The constant-current filtrometer system was used for measurement of erythrocyte deformability. Samples to be

measured were prepared as 10 mL of erythrocytes suspension and PBS buffer. The flow rate was held constant at 1.5 mL/min with an infusion pump. A 28 mm nucleopore polycarbonate filter with a 5  $\mu$ m pore diameter was preferred. A constant-current filterometer system was used in the measurement of the erythrocyte deformability. Pressure changes were detected by a pressure transducer while the erythrocytes passed through the filter and the data was transferred to computer with the help of MP 30 data equation systems (BIOPAC Systems Inc., Goleta, CA). The necessary calculations were performed with related computer programs by measuring the pressure changes at various times. Pressure calibration of the system was performed each time before measuring the samples. First buffer ( $P_T$ ) and then erythrocytes ( $P_E$ ) were passed through from the filtration system and the changes in pressure were measured. The relative refractory period value relative resistance (Rrel) was calculated by relating the pressure value of erythrocyte suspension to pressure value of buffer. The deformability index was interpreted as Rrel was increasing the ability of erythrocyte deformability was affected adversely.<sup>14</sup>

## Measurements of MDA levels and nitric oxide activities

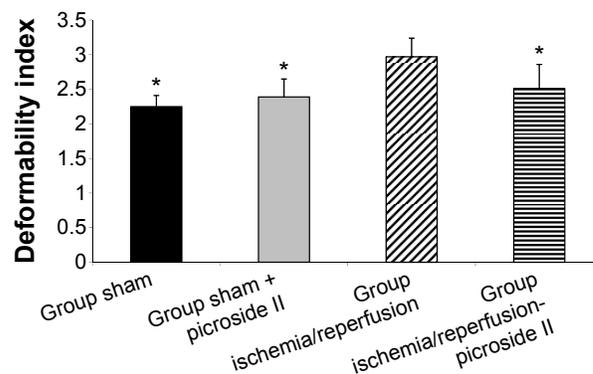
Esterbauer method was applied in order to measure lipid peroxidation. Malondialdehyde reacted with thiobarbituric acid at 90°C–95°C and resulted in pink chromogranin. After 15 minutes specimens rapidly cooled, then absorbances were read at 532 nm spectrophotometrically. Results were represented as nmol/g tissue protein.<sup>15</sup> Concentrations of stable oxidative metabolites of nitric oxide ( $\text{NO}$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ ) were measured in serum and NO production was determined. Measurement of nitrite concentration was performed with Griess reaction.<sup>16</sup>

## Statistical analysis

The statistical analyses were performed with SPSS 17.0 software program and  $P < 0.05$  was considered statistically significant. The findings were expressed as mean  $\pm$  standard deviation. The data were evaluated with Kruskal–Wallis variance analysis. The variables with significance were evaluated with Bonferroni corrected Mann–Whitney  $U$ -test.

## Results

Erythrocyte deformability as a significant determinant of relative resistance was increased in group I/R. Deformability index was significantly increased in the I/R group compared to groups sham, sham + picoside II, and I/R + picoside II



**Figure 1** Erythrocyte deformability index values of the groups.

**Notes:** Each bar represents the mean  $\pm$  SE. \* $P < 0.05$  compared to the group ischemia/reperfusion.

**Abbreviation:** SE, standard error.

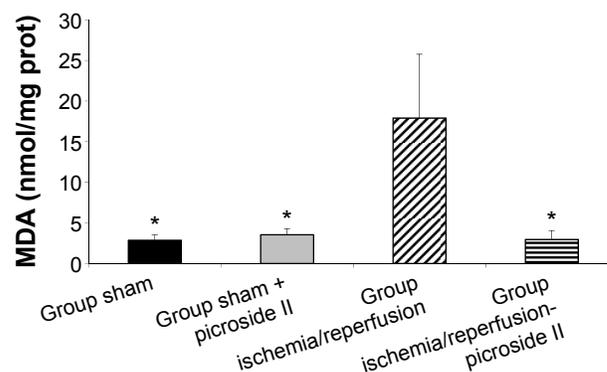
( $P < 0.0001$ ,  $P < 0.0001$ ,  $P = 0.007$ , respectively) (Figure 1). However, after picoside II treatment deformability index was similar with the control (groups sham, sham + picoside II, and I/R + picoside II).

Malondialdehyde (MDA) level was also higher in I/R group than the other groups ( $P < 0.0001$ ,  $P < 0.0001$ ,  $P = 0.007$ , respectively) (Figure 2). However, picoside II treatment in I/R-P group resulted in similar MDA levels to the sham, sham + picoside II groups (Figure 2).

NO activity was also higher in I/R group than the other groups ( $P = 0.015$ ,  $P = 0.020$ ,  $P = 0.016$ , respectively) (Figure 3). However, picoside II treatment in I/R + picoside II group resulted in similar NO activity to the sham, sham + picoside II groups (Figure 3).

## Discussion

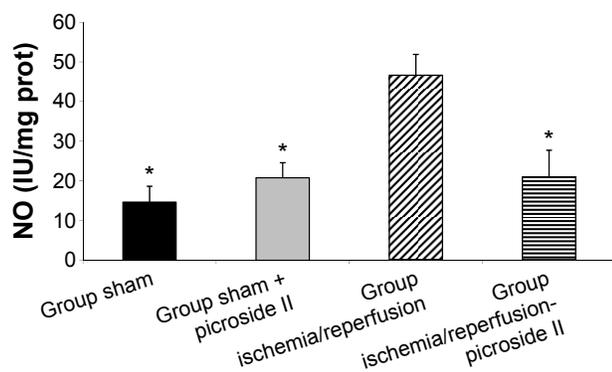
Picoside II ( $\beta$ -D-glucopyranoside, 1a, 1b, 2, 5a, 6, 6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxy]-1-a(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl)



**Figure 2** Malondialdehyde (MDA) levels of the groups.

**Notes:** Each bar represents the mean  $\pm$  standard error. \* $P < 0.05$  compared to the group ischemia/reperfusion.

**Abbreviation:** prot, protein.



**Figure 3** Nitric oxide (NO) activity values of the groups.

**Notes:** Each bar represents the mean  $\pm$  standard error. \* $P < 0.05$  compared to the group ischemia/reperfusion.

is a major iridoid glycoside isolated from *P. scrophularii-flora* Pennell (Scrophulariaceae).<sup>17</sup> Previous studies have shown that picoside II has a number of pharmacological effects, including neuroprotective,<sup>12,18</sup> hepatoprotective,<sup>19</sup> antiapoptotic, anticholestatic, anti-inflammatory, and immune-modulating activities.<sup>10,11</sup> In addition, picoside II is also reported to possess potent antioxidant activities.<sup>9,18</sup>

Unpaired electrons, free radicals, are highly reactive and readily take part in chemical reactions with virtually all cell components (lipids, proteins, complex carbohydrates and nucleic acids) in the body. These reactions occur through a chain of oxidative reactions to cause tissue injury. For most biological structures (like lipids, proteins, and nucleic acids), free radical damage is closely associated with oxidative damage, causing direct cellular injury by inducing lipid and protein peroxidation and damaging nucleic acids.<sup>20</sup> The highly unsaturated fatty acids present in cellular membranes are the most susceptible macromolecules to oxidative damage in cells. Endothelial cells, macrophages, neutrophils, and neuronal cells generate superoxide ( $O_2^-$ ) and NO, which can combine to form peroxynitrite anion ( $ONOO^-$ ). Peroxynitrite, known to oxidize sulfhydryls and to yield products indicative of hydroxyl radical ( $\cdot OH$ ) reaction with deoxyribose and dimethyl sulfoxide, is shown herein to induce membrane lipid peroxidation.<sup>21</sup> Lipid peroxidation is a free-radical initiated chain reaction resulting in sequential abstraction of hydrogen ions from polyunsaturated fatty acids. Lipid peroxides, such as MDA, are markers and intermediate products of lipid peroxidation and are used for the assessment of tissue injury made by the free radicals produced by I/R.<sup>22</sup>

Zhang et al<sup>23</sup> animal experiment showed that the amount of MDA, NO, and  $H_2O_2$  can directly reflect the changes of the free radicals content in vivo in cerebral ischemic injury. The amount of MDA, NO, and  $H_2O_2$  increased

significantly in serum and brain tissue after establishing the animal models and reduced after injecting picoside II intraperitoneally. It was suggested that picoside II can enhance the activity of endogenous antioxidant enzymes and clear the excess oxygen radicals to protect brain tissue from oxidative injury.

Wu et al<sup>24</sup> study showed that picoside II remarkably increased the amount of NO in ischemic myocardium. Therefore, it is speculated that picoside II improves postischemic cardiac functional recovery as a result of an increase in NO production, because NO can reinforce myocardial contractility.<sup>25,26</sup>

Erythrocytes must be able to extend and curve and have the capability to move in end organ capillaries to deliver the oxygen and vital molecules to the tissues and clear the metabolic wastes via final organ capillaries. This ability is termed as “deformability” and gets more important in microcirculation. Altered erythrocyte deformability is not only important for oxygen delivery capacity of the erythrocytes but it is also critical for the survival of the circulating erythrocytes.<sup>27–29</sup>

Wu et al<sup>24</sup> showed conclusively that picoside II pretreatment significantly increased the values of left ventricular developed pressure, positive first-order derivative of ventricular pressure (+dp/dt), negative first-order derivative of ventricular pressure (–dp/dt), and coronary flow, and decreased the size of infarction, which suggests that picoside II could produce a protective effect on myocardial ischemia-reperfusion injury in rats.

All these data and our findings indicate that erythrocyte deformability is impaired in rats subjected to I/R and this impairment leads to disturbance of microvascular perfusion and related problems. Hence, we think that measurement of erythrocyte deformability can be useful as a parameter in cases of I/R. We also observed beneficial effect of picoside II on maintaining erythrocyte deformability during periods of I/R, but we still think these promising results should further be supported by more detailed studies with larger numbers.

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## Disclosure

The authors report no conflicts of interest in this work.

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