Effect of curcumin on erythrocyte deformability in mice with limb ischemia-reperfusion injury

Gökhan Arslan, Hakan Kartal, Faruk Metin Çomu, Erdem Çetin, Hüseyin Sicim, Ertan Demirdaş, Celalettin Günay, Bilgehan Savaş Öz

1Department of Cardiovascular Surgery, University of Health Sciences, Gülhane School of Medicine, Ankara, Turkey
2Department of Cardiovascular Surgery, Gülhane Training and Research Hospital, Ankara, Turkey
3Department of Physiology, Kirikkale University Faculty of Medicine, Kirikkale, Turkey
4Department of Cardiovascular Surgery, Karabük University Faculty of Medicine, Karabük, Turkey

ABSTRACT

Objectives: This study aims to examine the impact of curcumin on erythrocyte deformability in a mouse model that can be used for short-term ischemia/reperfusion (IR) injury.

Materials and methods: A total of 30 mice were divided into five groups of six animals in each. The mice were subjected to unilateral hind limb ischemia on the femoral vessels for 2 h. Reperfusion was achieved for the next 2 h. Curcumin (100 mg·kg⁻¹) was administered intraperitoneally (CUR and IR/CUR groups). The mice were euthanized by intraperitoneal administration of ketamine (100 mg·kg⁻¹) and blood was drawn from the heart. A constant flow filter system was used to assess erythrocyte deformability.

Results: There was a statistically significant difference in the mean deformability index among the groups. The IR group (2.1±0.3) significantly higher than the mean of control group (1.6±0.2), CUR group (1.6±0.1) and DMSO group (1.6±0.2). The deformability index values of the IR/CUR group approach the control group through the application of the curcumin (1.8±0.2).

Conclusion: Our study showed negative effects of IR on erythrocyte deformability in mice. Also, curcumin had positive effects by reversing the undesirable effects of IR.

Keywords: Curcumin, erythrocyte deformability, ischemia-reperfusion.

Erythrocyte that primary function enables respiration in tissues by providing oxygen and removing carbon dioxide, possess a unique biconcave form supplied by the superior construction of the membrane-skeleton system. During the lifespan of an erythrocyte, it circulates between arteries, veins, and small capillaries. Erythrocyte deformability, the erythrocyte’s capacity to change shape, is essential for valid entry through these vessels. Deformability is affected by pH value, free radicals, surface/volume ratio, viscosity, hemoglobin concentration, and metabolic processes controlling adenosine-5’-triphosphate (ATP) levels. Failure to sustain deformability results in a shortening of erythrocyte life.

Blood vessel occlusions of various origins have revealed that acute ischemia and subsequent reperfusion are a catastrophic process, leading to critical tissue damage combined with the end-organ loss of function. Hind limb ischemia model on mice is the most commonly used preclinical model for limb ischemia/reperfusion (IR) and peripheral arterial diseases. Acute ischemia of a limb ensues in several stages, and oxygen deficiency is the event’s primary contribution. Studies in the literature have...
shown that metabolic processes and free radicals affect erythrocyte deformability changes during the IR process. Consequently, erythrocyte deformability and comprehension of the cycles in charge are essential for picking the ideal helpful technique. Analyzing erythrocyte deformability may provide novel therapeutic approaches for lessening the negative effects of IR on erythrocytes.

Curcumin is a yellow-orange polyphenolic compound extracted from the rhizome of turmeric plant *Curcuma longa*. It is used as a colorant and spice in foods, which belongs to the Zingiberaceae family. Curcumin is a multifunctional compound from a natural plant and has various therapeutic activities, such as anti-inflammatory, antioxidant, anticoagulant, anti-carcinogenic, anti-bacterial, and wound-healing. The effects of curcumin to prevent tissue injury due to IR has been well documented in the literature. However, curcumin's effects on the deformability of erythrocytes related to IR injury have not yet been examined, yet.

In the present study, we aimed to create a hind limb IR mouse model using an atraumatic vascular bulldog clamp on the right femoral artery and to investigate the effect of curcumin on erythrocyte deformation during IR in mice.

**MATERIALS AND METHODS**

**Animals and experimental protocol**

In this experimental study, a total of 30 male Swiss albino mice weighing 45 to 65 g (3- to 4-month-old) were used in the Physiology Laboratory of the Kirikkale University and Gazi University Laboratory Animal Breeding and Experimental Researches Center. All mice were randomly divided into five groups of six mice in each group. They were housed in groups of six in clean cages with a 12-h light/dark cycle at 24±2°C temperature and 60±5% humidity. They were allowed to access food and water ad libitum, until 2 h before the induction of anesthesia. All endeavors were made to limit the number of animals utilized and animal languishing. The study protocol was approved by the Gazi University Ethics Committee of Experimental Animals (No: 66332047). All procedures were conducted in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.

The animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) co-administered with 100 IU/kg of heparin. Anesthesia was continued by an additional half dose every 90 min. The subjects were placed with the ventral side up and fixed the feet using a tape to expose the upper thigh area. Hairs in the groin and upper thighs of the mice were removed gently with the help of a lancet. After making a 1.5-cm incision using skin scissors running parallel to the right inguinal ligament, adipose tissue was dissected away from the femoral region. The right femoral artery was isolated by using a bulldog clamp.

**Control group**

The right femoral artery was surgically isolated; however, mice were not subjected to any IR. After 4 h, a 0.5-mL blood sample was taken by cardiac puncture and the mice were sacrificed.

**Curcumin (CUR) group**

Curcumin powder (Sigma Aldrich, MA, USA) was dissolved in dimethyl sulfoxide (DMSO) (11 mg/mL) to prepare a solution. Similar steps were followed. Curcumin was given (100 mg/kg) intraperitoneally. After 4 h, a 0.5-mL blood sample was taken and the mice were sacrificed finally.

**DMSO group**

Dimethyl sulfoxide, known as DMSO (1 mL/kg, 10% solution in saline), is a commonly used solvent for polar and non-polar small molecules. This group was created to find out whether the solvent solution had a positive or negative contribution to the effect of curcumin on the erythrocyte deformability. The DMSO was given (curcumin is soluble in 11 mg/mL DMSO) intraperitoneally. Similar steps were followed. Blood samples were collected after 4 h of follow-up.

**IR group**

The right femoral artery was isolated for 2 h using a bulldog clamp. After removing the clamp, reperfusion was implemented for another 2 h. Finally, the mice were sacrificed, and approximately 0.5 mL of blood samples were stored for later analysis.

**IR/CUR group**

The right femoral artery was isolated using a bulldog clamp for 2 h, as in the IR group. In addition, curcumin was administered intraperitoneally (100 mg/kg), starting simultaneously with the reperfusion period after removing the clamp. At the end of the 2-h reperfusion period, the mice were sacrificed by taking blood from the heart.
Effect of curcumin on erythrocyte deformability

Erythrocyte deformability measurements

Only about 0.5 mL of blood can be collected from mice by intracardiac puncture. Blood was centrifuged at 1,000 rpm for 10 min as quickly as possible to prevent hemolysis of erythrocytes. Serum was eliminated, despite the buffy coat on the erythrocytes. An isotonic phosphate-buffered saline (PBS) was used to be added to the collapsing erythrocytes and the preparation was centrifuged at 1,000 rpm for 10 min. The pure red cell packs were then, obtained from the three rewashing cycles. Red blood cell packs were blended with PBS to form a suspension with a 5% hematocrit prediction. A total of 10 mL of erythrocyte suspension was formed by the addition of PBS at 22°C. These red cell suspensions were used for the analysis of deformability. A standard flow filter system was used to evaluate erythrocyte deformability. The flow rate was kept constant at 1 mL/min with the infusion pump once. A 28-mm nucleoporin polycarbonate filter with a pore diameter of 5 μm was preferred. As the red blood cells passed through the filter, pressure changes were determined with a pressure transducer and the results were conveyed to the PC using an MP30 data equation system (Biopac® Systems Inc., Commat, USA). Calculations were made by measuring voltage changes with the relevant PC applications. The pressure calibration of the process was checked before each sample measurement. The buffer (PT) and erythrocytes (PE) were filtered and calculated by recording the changes in pressure. The relative refractory period value (Rrel) was determined by correlating the pressure estimation of the erythrocyte suspension and the pressure estimation of the buffer solution. It was concluded that an Rrel increase in the deformability index negatively affected the deformability of erythrocytes.[6]

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). Continuous variables were presented in mean ± standard deviation (SD). The compliance of the data to normal distribution was evaluated using the Shapiro-Wilk test. Variance homogeneity was evaluated by the Levene’s test and it was found to meet these assumptions. The difference between the group averages was examined using one-way analysis of variance (ANOVA). In case of a significant difference, Bonferroni correction was used as a post-hoc test to find the source of the difference. Its clinical significance was determined according to the partial eta-squared ($\eta^2$) and ANOVA breakpoints (0.0099 small, 0.0588 medium, and 0.11379 large effects) as recommended by Cohen.[17] The effectiveness of treatment applied to the IR group was examined using the Pearson correlation coefficient ($r$). A $p$ value of <0.05 was considered statistically significant.

RESULTS

The mean deformability index of mice was 1.732±0.260. As a result of one-way ANOVA, a statistically significant difference was found in the means of deformability index among the five groups (F (4.25)=7.045, $p=0.001$) (Table 1). The mean deformability index was significantly higher in the IR/CUR group than the average of IR group (2.08±0.25), control group (1.59±0.20), CUR group (1.59±0.11), and DMSO group (1.63±0.22).

Clinical significance for ANOVA was evaluated by partial eta-squared ($\eta^2$). It showed a high effect level ($\eta^2=0.530>0.1379$) compared to the limit values (0.0099 small, 0.0588 medium and 0.1379 large effects) as proposed by Cohen.[17] The Pearson correlation coefficient ($r$) obtained for the effectiveness of the curcumin usage applied to the IR group was higher than the Cohen’s recommended limit values (0.10 small, 0.30 medium and 0.50 large effect) ($r=0.45>0.30$), showing an almost level effect (Table 1).

Figure 1 and 2 show deformability index distributions of five groups. The mean deformability index of the IR group was significantly higher,

Table 1. Comparison of deformability index of the groups

<table>
<thead>
<tr>
<th></th>
<th>Control (1)</th>
<th>DMSO (2)</th>
<th>CUR (3)</th>
<th>IR (4)</th>
<th>IR/CUR(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Deformability index</td>
<td>1.59±0.20</td>
<td>1.63±0.22</td>
<td>1.59±0.11</td>
<td>2.08±0.25</td>
<td>1.78±0.15</td>
</tr>
<tr>
<td>Test statistics</td>
<td>F(4,25)=7.045*</td>
<td>p=0.001**</td>
<td>$\eta^2=0.530$</td>
<td>1-4 (p=0.002)</td>
<td>2-4 (p=0.004)</td>
</tr>
<tr>
<td>Source of difference‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DMO: Dissolved in dimethyl sulfoxide; CUR: Curcumin; IR: Ischemia/reperfusion; SD: Standard deviation; * One-way ANOVA test statistical value; ** Statistically significant at 0.05 significance level (p<0.05); † Partial Eta-squared effect size value; ‡ Bonferroni correction was used as post-hoc test.
compared to the other groups. After curcumin administration, the mean values of the IR/CUR group, whose deformability index decreased, approached the control group.

DISCUSSION

In this study, we examined the effect of curcumin use on erythrocyte deformability in experimental lower extremity IR injury in mice. Our results showed that the curcumin was useful to preserve the normal structures and enhanced erythrocyte deformability performance by reducing the adverse effects of the post-IR injury.

Ischemia/reperfusion is a pathological condition in which the biochemical and physiological changes occurring in blood vessels play an essential role in deteriorating microcirculation homeostasis. The stability between blood flow and tissue perfusion takes place depending on the rheological properties of the blood. These properties are related to hematocrit, plasma viscosity, erythrocyte aggregation, and erythrocyte deformability, which play a role in determining blood viscosity. The deformability properties of erythrocytes determine the geometry of erythrocytes, the rheological properties of intracellular fluid, and the structural and functional integrity of lipids and proteins in the membrane structure. One of the most critical factors affecting the structural and functional integrity of cells is oxidative stress.

After IR injury, the increase in the amount of oxidant substance causes oxidative damage by disrupting the balance between the oxidant-antioxidant arrangement. Membrane lipids, which have an essential role in protecting the structural and functional integrity of erythrocytes, are peroxidized with increasing free radicals in IR damage and lose their deformability properties consequently. This loss in erythrocytes causes the tissues not to be oxygenated sufficiently and, accordingly, the process leading to organ failure begins.

Curcumin, a yellow-orange part of turmeric powder, is a standard polyphenol product derived from the Curcuma longa plant. For hundreds of years, curcumin has been used in medical preparations or as a natural food coloring ingredient. Curcumin is a phytochemical that positively affects various physiological and cellular processes and leads to therapeutic effects against different diseases. In recent years, extensive in vitro and in vivo studies have shown that curcumin has anti-amyloid, anti-cancer, antiviral, antioxidant, and anti-inflammatory properties. Feasible underlying molecular mechanisms have been proposed and appear to comprise a number of molecular ambitions, such as transcription factors and inflammatory cytokines. Consequently, besides the effectiveness and regulation of more than one objective and its safeguard for human use, curcumin has acquired tremendous attention as a capable therapeutic agent in many malignant illnesses, arthritis allergies, Alzheimer’s disease, and different inflammatory processes.
In recent years, studies on the antioxidant effects of curcumin have gained increasing attention. Anti-inflammatory molecules such as curcumin directly affect the erythrocytes, platelets, and plasma proteins. Several studies have shown that curcumin protects cells against oxidative damage. However, review of the literature using curcumin, erythrocyte deformability, and oxidative damage as keywords reveals no study at the time of the conduct of this study. We focused on the changes that erythrocyte deformability due to IR injury. In this study, we showed that curcumin had a protective effect on erythrocyte deformability in lower extremity IR injury in mice similar to the control group. Preventing erythrocyte deformability damage is essential for improving outcomes in all circulation-related diseases. Therefore, treatment or pre-treatment with curcumin may have promising applications due to the ability of this preparation to maintain the elasticity and membrane stiffness of the erythrocytes and, thus, assist in deforming the erythrocytes under the action of mechanical force. Numerous experimental evidence support the cell-protective properties of curcumin; however, the mechanisms by which curcumin exerts antioxidant effects are still unclear.

The limitations of this study merit consideration. In particular, mice were included in the study, as the erythrocyte deformability measurement method that we used can be practiced with very small amounts of blood. One additional potential burden is the low amount of blood from mice that limits the examination of other parameters to confirm our study results.

In conclusion, this study findings suggest that erythrocyte deformability is significantly altered in lower limb IR injury in mice. Also, curcumin has beneficial effects on preserving erythrocyte deformability during IR. Based on these results, we can speculate that measurement of erythrocyte deformation is of utmost importance to identify the presence and extent of the inflammation and to examine these parameters during novel medical treatments. Nonetheless, further studies, particularly in animal models involving varying biochemical parameters, are warranted. The findings of this study need to be supported in the future by well-designed animal and human studies.

Declaration of conflicting interests
The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding
The authors received no financial support for the research and/or authorship of this article.

REFERENCES


