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ORIGINAL ARTICLE

Preventive Effect of Edaravone Against Ischemia-Reperfusion Injury in Rat Epigastric Island Flaps: An Experimental Study

Sıçan Epigastrik Ada Fleplerinde Edaravonun İskemi-Reperfüzyon Hasarına Karşı Koruyucu Etkisi: Deneysel Bir Çalışma

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ABSTRACT

Objective: Ischemia-reperfusion (I/R) injury is tissue damage that occurs when blood returns to a tissue after an ischemic phase. Reperfusion initiates a cascade of acute inflammatory processes that end in cell death, tissue malfunction, and necrosis. Edaravone (3-methy-1-pheny1-2-pyrazolin-5-one) is a powerful and unique synthetic radical scavenger. This research investigated the effects of edaravone on I/R damage.
Material and Methods: Sixteen adult male Sprague–Dawley rats were utilized. Eight rats were allocated at random into two groups. Group 1 (the control group) experienced ischemia and reperfusion of an abdominal skin flap for 10 hours without therapy. Group 2 (treatment group) received an intraperitoneal injection of 3 mg/kg edaravone 10 hours prior to ischemia and reperfusion in an abdominal skin flap. Using planimetry, the flaps were examined at intervals of one, three, seven, and 10 days. Then, the flaps were removed for biochemical (measurement of tissue glutathione (GSH), tissue protein, lipid peroxidation (MDA), nitric oxide (NO) levels) and histopathological investigation (measurement of tissue glutathione (GSH), tissue protein, lipid peroxidation (MDA), and nitric oxide (NO) levels).
Results: We found no significant changes ($p > 0.05$) between groups 1 and 2 in terms of NO, MDA, GSH, or planimetric analyses. Group 2 had a lower neutrophil count than group 1, but the difference was not statistically significant ($p > 0.05$).
Conclusion: Edaravone is a highly effective antioxidant. Nevertheless, our research indicates that it may not influence I/R damage in a skin-flap model.

Key words: epigastric flap, edaravone, ischemia reperfusion injury

ÖZ

Amaç: İskemi-reperfüzyon (I/R) hasarı, iskemik fazdan sonra kan dokuya geri döndüğünde meydana gelen doku hasarıdır. Reperfüzyon, hücre ölümü, doku bozukluğu ve nekroz ile sonuçlanan bir dizi akut inflamatuar süreç başlatır. Edaravone (3-meth-1-pheny1-2-pyrazolin-5-one) güçlü ve benzersiz bir sentetik radikal temizleyicidir. Bu araştırmada, edaravonun I/R hasarı üzerindeki etkileri araştırıldı.
Gereç ve Yöntemler: 16 yetişkin erkek Sprague-Dawley sıçanı kullanıldı. Sekiz sıçan rastgele iki gruba ayrıldı. Grup 1 (kontrol grubu), tedavi olmaksızın 10 saat boyunca karın derisi flebine iskemi ve reperfüzyonu uygulandı. Grup 2'ye (tedavi grubu) iskemiden 10 saat önce intraperitoneal 3 mg/kg edaravon enjeksiyonu ve karın derisi flebinde reperfüzyon uygulandı. Planimetri kullanılarak flepler 1, 3, 7 ve 10 günlük aralıklarla incelendi. Daha sonra biyokimyasal (doku glutatyonu [GSH], doku proteini, lipid peroksidasyonu [MDA] ve nitrik oksit [NO] düzeylerinin ölçümü) ve histopatolojik (doku glutatyonu [GSH], doku proteini, lipid peroksidasyonu ölçümü) için flepler çıkarıldı. MDA] ve nitrik oksit [NO] seviyeleri)
Bulgular: Grup 1 ve 2 arasında NO, MDA, GSH veya planimetrik analizler açısından anlamlı bir değişiklik ($p > 0.05$) bulamadık. Grup 2, grup 1'den daha düşük nötrofil sayısına sahipti, ancak fark istatistiksel olarak anlamlı değildi ($p > 0.05$).
Sonuç: Edaravone çok etkili bir antioksidandır. Yine de araştırmamız, bir deri flep modelinde I/R hasarını etkilemeyebileceğini gösteriyor.

Anahtar Kelimeler: edaravon, iskemi reperfüzyon hasarı, epigastrik flap

Introduction

In ischemia and subsequent reperfusion, oxygen penetrates the tissues, and free oxygen radicals are produced. The resulting damage is known as ischemia-reperfusion (I/R) injury. The interaction between the free oxygen radicals and lipid acid radicals in the damaged cell membrane leads to lipid peroxidation (1). Increased lipid peroxidation results in the formation of MDA, edema, and cell expansion. Consequently, DNA chain breaks and cell necrosis ensue (2). It is well known that I/R degradation contributes significantly to the loss of free flaps. Acute I/R injury comprises cell apoptosis caused by the interaction between leukocytes and endothelial cells following the cessation of microvascular bleeding and the release of reactive oxygen types (3). Natural antioxidants defend the body against free radicals and prevent oxidative stress and associated diseases. Accordingly, they play an essential role in human health (4).

Edaravone (3-methy-1-pheny1-2-pyrazolin-5-one) is a powerful and unique synthetic radical scavenger. In addition to inhibiting nonenzymatic lipid peroxidation and the lipoxygenase pathway, this medication has substantial antioxidant effects against I/R-induced vascular endothelial cell damage and delayed neuronal death, brain edema, and accompanying neurological impairments (5-8). In 2002, edaravone was licensed for use in Japan to treat acute

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brain infarctions, and it has been found to have neuroprotective properties against stroke (5,7).

Using a skin flap model in rats, we investigated the impact of edaravone on reperfusion injury in the light of the aforementioned results.

Materials and Methods

Animal model

This research included 16 male Sprague–Dawley rats weighing 230–270 g. The animals were housed in single-filter cages in separate rooms at a temperature of 24°C with a 12-h light/dark cycle. During the research period, they had free access to drink and food, but only water was provided six hours prior to surgical operations (ethics number: 03220116).

Experimental protocol

The features of the experimental protocol were as follows:

-The animals were divided into two groups, each containing eight rats.

-In the edaravone group, after intraperitoneal edaravone application, the flap was elevated, and ischemia was induced.

-In the control group, the flap was raised, and ischemia was caused without any therapy.

-24 hours following reperfusion, samples were collected for biochemical examination.

-On the tenth postoperative day, the rats were slaughtered, the flaps were imaged for survival assessment, and histological samples were collected.

Surgical procedures

All surgical operations were conducted by the same surgeon (OA). After administering xylazine (15 mg/kg; Alfazyne®, Alfasan International BV, Netherlands) and ketamine (50 mg/kg; Ketalar®, Pfizer Warner-Lambert, Turkiye) intramuscularly, the abdomen and inguinal regions of the rats were shaved. Antisepsis was accomplished by providing cetrimide and chlorhexidine gluconate (Savlon®, GlaxoSmithKline, Brentford, UK). The animals were then put in the supine posture on the operating table. The 6x4 cm2 epigastric flap was planned and demarcated with a skin marker. Intraperitoneal edaravone (3 mg/kg) was delivered to the rats in the edaravone group (group 2). Thirty minutes after the edaravone injection, the flap, comprising the subcutaneous and superficial fascia at the distal portion of the inguinal ligament, was raised craniocaudally. The epigastric artery and vein were then identified and separated from the surrounding tissue. The pedicle was secured with an Acland V2 micro clamp for 10 hours (Fig. 1). After 10 hours, the clamp was withdrawn and peristalsis was used to reestablish arterial–venous flow. The flap displayed an increase in temperature and a change in color before being repositioned with 4–0 polypropylene sutures. In group 1 (the control group), the flap was raised untreated. Under general anesthesia and analgesia with intramuscular ketamine and xylazine, 1x1 cm2 tissue samples were obtained from the same sides of all flaps 24 hours following reperfusion for biochemical evaluation. After 10 days, the rats were killed with a lethal dosage of ketamine, and images were taken to quantify the flap's surviving area. After images were taken on the tenth day, 1x1 cm2 samples were extracted from the same sides of all flaps for histological measurements so that the necrotic region would not be altered.

Macroscopic measurement of the surviving flap area

On postoperative day 10, the flaps were photographed from a distance of 25 cm using a Canon EOS 600d camera, and the surviving flap area was computed using the Image-Pro Express application (Version 6.0, Media Cybernetics, USA). The size of the surviving flap was determined by subtracting the necrotic region from the overall flap area.

Histopathological and biochemical analysis

Evaluation of lipid peroxidation in tissue (MDA)

Tissue samples (about 100 mg) were analyzed for lipid peroxidation by measuring levels of malondialdehyde (MDA) as thiobarbituric acid reactive substances (TBARS) (9). Briefly, trichloroacetic acid and TBARS reagent were added to tissue samples, which were then combined and incubated for 60 minutes at 100 °C. After chilling the samples on ice, they were centrifuged at 3000 rpm for 20 minutes, and the absorbance of the supernatant was measured at 535 nm. MDA levels were determined using tetraethoxypropane and the standard calibration curve and are reported as nanomoles per gram of protein.

Evaluation of tissue glutathione (GSH) concentrations

Following Ellman's approach (10), the GSH content of tissue samples (about 100 mg) was measured spectrophotometrically. During this procedure, thiols react with 5.5'-dithiobis-(2-nitrobenzoic acid) to produce a colored anion with a maximum peak at 412 nm. GSH concentrations were determined using a standard calibration curve and are reported as nmol/mg protein.

Nitric oxide assay

At 24th hour, around 100 mg of tissue was extracted from the distal portion of the muscle and skin paddle. Abcam's nitric oxide test was used with the Griess reagent. At 540 nm, absorbance was measured spectrophotometrically. The tissue nitric oxide concentrations were reported in micromoles per milligram of tissue.

Peripheral neutrophil count

At 24th hour, peripheral blood was collected for Wright–Giemsa-stained smears to measure the circulating neutrophils. Neutrophils were manually counted in five random fields per slide at 200x high-power-field magnification. The circulation neutrophil count was derived by multiplying the average value by 1.000.

Histopathological examination

The histopathological studies were conducted based on Ersel et al.' (11) approach. After 24-hour fixation in 10% buffered formalin, normal paraffin wax embedding and blocking procedures were performed on the skin biopsy samples. Using a Leica RM2145 microtome (Germany), slices of about 5 m were cut. The sections were then stained with hematoxylin and eosin and Mallory's azan (MA) stains. Necrosis, edema, polymorphonuclear leukocyte (PMNL) infiltration, and vascularization were assessed using a light microscope (Olympus BX-51 light microscope, Olympus C-5050 digital camera) and modified Verhofstad scoring on sections stained with hematoxylin and eosin and Mallory azan (MA) (Table 1). Anti-inducible nitric oxide synthase (iNOS) antibody was used to investigate immunohistochemistry expression. To suppress endogenous peroxidase activity, paraffin slices were submerged in xylene overnight and then treated in 3% H2O2 methanol. The sodium citrate solution was cooked in a microwave at 90 W for 5 minutes and at 360 W for 15 minutes. The sections were subsequently treated for 24 hours at 4 °C with the primary antibody (anti-iNOS; 1/100). Antibodies were detected using a Histostain-Plus Bulk Kit (Bioss, Inc.) against rabbit immunoglobulin G, and 3.3' diaminobenzidine was used to view the final result. The immunoreaction was observed by light microscopy (Olympus BX-51 light microscope, Olympus C-5050 digital camera) at 40x magnification.

Table 1: Histopathologic evaluation Verhofstad modified scoring table.

Score	Necrosis	Edema	PMNL*	Vascularization
0	None	None	Normal	None
1	Superficial	Light	Light	Light
2	Pronounced	Pronounced	Pronounced	Pronounced
3	Massive	Dense	Massive	Dense

* PMNL-polymorph nuclear leukocytes

Statistical analysis

Statistical evaluation was performed using a one-way analysis of variance (ANOVA). The differences among groups regarding flap survival area, modified Verhofstad score, GSH, NO, and MDA levels, and tissue neutrophil count were evaluated. When the p-value from the variance analysis was statistically significant, the post hoc Tukey test was conducted. Results are presented as mean ± SEM. A value of $p < 0.05$ was considered significant.

Results

No rats died, and no complications were observed during the study.

Flap survival area

The necrotic region was deducted from the overall flap area to determine the flap survival area (23.4 cm2). The mean survival area in the control group was 22.43±1.06 cm2 while in the edaravone group it was 22.31±1.10 cm2. The difference between the groups was not significant ($p > 0.05$).

Biochemical evaluation

Tissue levels of GSH, MDA, and NO in the control group were 1.4±0.9 nmol/g, 116.2±12.7 nmol/g, and 19.5±4.07 U/g, respectively. The mean GSH, MDA, and NO levels in the edaravone group were 1.5±0.3 nmol/g, 118.6±11.2, and 20.2±8.5 U/g, respectively. There were no statistically significant differences between the groups for any of these indicators ($p > 0.05$) (Table 2).

Table 2: Average flap survival, GSH, MDA, NO values and neutrophil count results of control and edaravone groups.

Groups	Flap Survival(cm^2)	GSH (nmol/gr tissue)	MDA (nmol/gr tissue)	NO (U/gr tissue)	Neutrophil Count(cells/µl)
Control	22,43± 1,06	1.4 ± 0.9	116.2± 12.7	19.5± 4.07	20360 ± 1850
Edaravone	22,31± 1,10	1.5 ± 0.3	118.6 ± 11.2	20.2 ± 8.5	19650 ± 2350

Histopathological examination

Necrosis, edema, PMNL infiltration, and vascularization were evaluated in samples taken from 16 subjects (8 each from the treatment and control groups). As mentioned, the values for these parameters were similar between groups. The necrosis values for the 16 subjects varied between 0 and 3, being 2.5 ± 0.5 for the control group and 2.3 ± 0.5 for the treatment group. Thus, there was no significant difference in the necrosis values of the control group and those of the treatment group with edaravone administration. Edema, PMNL infiltration, and vascularization scores in the control group were 2.75 ± 0.4, 2.25 ± 0.4, and 1 ± 0.5, respectively. In the edaravone group, the mean edema, PMNL infiltration, and vascularization scores were 2.1 ± 0.9, 2 ± 0.7, and 1.1 ± 0.6, respectively. These differences were not statistically significant ($p > 0.05$) (Table 3). The mean neutrophil counts were 20.360 ± 1850 cells/µl in the control group and 19.650 ± 2350 cells/µl in the edaravone group. Statistically significant differences were not observed between the groups ($p > 0.05$) (Table 2).

Table 3: Average values of histopathological scores.

Groups	Necrosis	Edema	PMNL*	Vascularization
Control	2.5± 0.5	2.75 ± 0.4	2.25± 0.4	1±0.5
Edaravone	2.3± 0.5	2.1 ± 0.9	2 ± 0.7	1.1 ±0.6

* PMNL-polymorph nuclear leukocytes

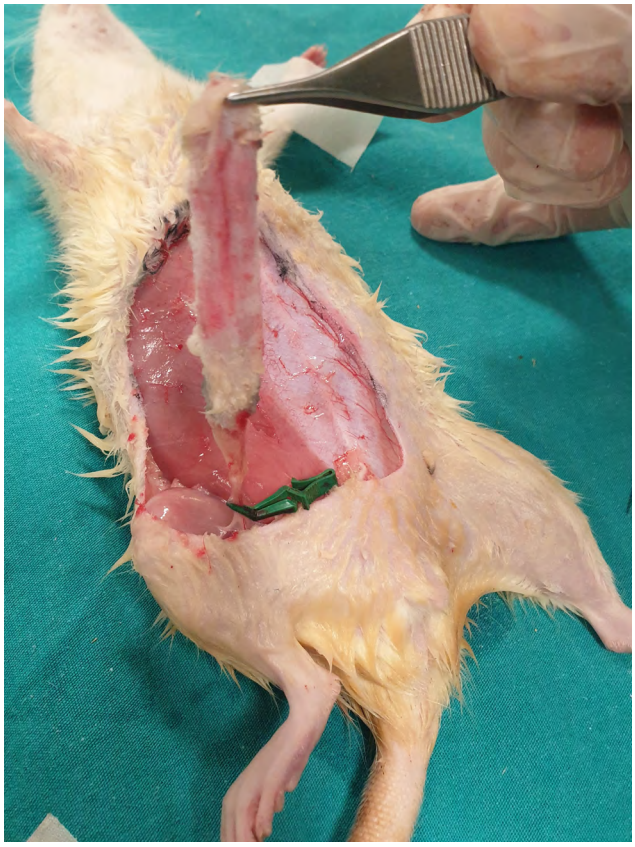

Fig. 1 After flap elevation, the pedicle was clamped to create ischemia

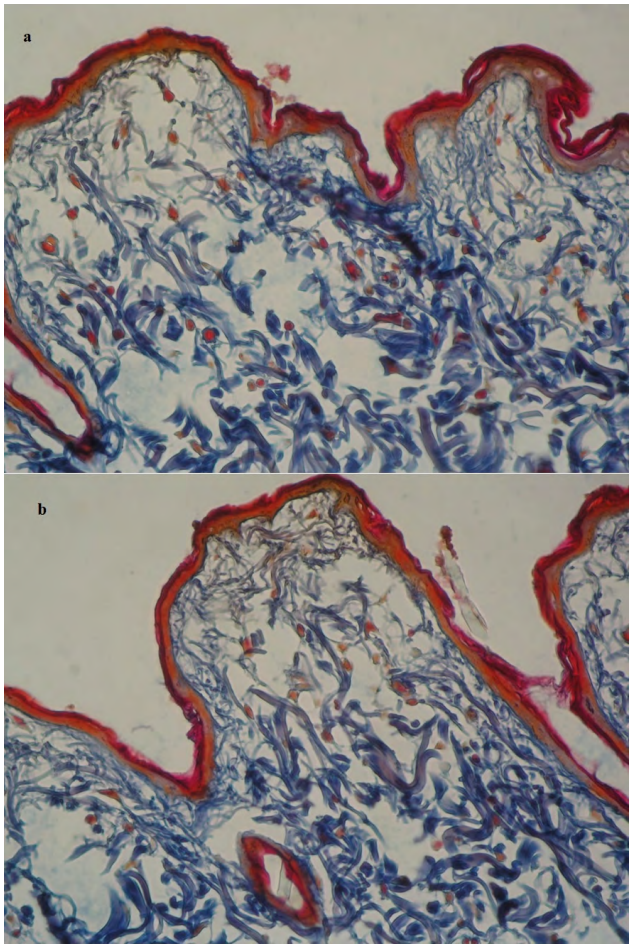

Fig. 2 Control group (a) and edaravone group (b) histopathological views; Mallory's azan (MA) staining, original magnification × 10

Discussion

I/R injury refers to tissue damage caused by the return of blood to a tissue after a period of ischemia. Reperfusion sets off a cascade of acute inflammatory processes, resulting in cellular death and, therefore, tissue malfunction and necrosis (12). Regardless of a surgeon's skill, post-revascularization I/R damage is an unavoidable pathophysiological process (13). The majority of investigations on I/R damage have been done on rats (14-18), and thus rats have served as the model animal in a number of studies. Yoshida et al. (19) analyzed the effects of vitamin C and mannitol on epigastric rat flaps and reported that, from a therapeutic standpoint, epigastric artery flaps might be utilized to model free flaps and severed fingers rather than epigastric island flaps. Therefore, in the current investigation, a flexible epigastric flap was used. Biochemical, histological, and macroscopic consequences occur 6–10 h after reperfusion in rat models according to the research on I/R damage (20-22). In this investigation, the duration of ischemia was 10 hours. A recent study found that antioxidants are highly effective when supplied before I/R damage occurs (23). Accordingly, in the current investigation, the therapy was delivered before the surgical procedure.

The potential therapeutic advantages of edaravone have been identified, and it is now being studied. Oxygen free radicals have been implicated in the etiology of I/R injuries, such as cerebral infarction (5,7,24); hence, they are being researched for I/R injuries, including cerebral infarction. A recent Japanese randomized multicenter clinical study (8) indicated that edaravone delivered within 72 hours after the beginning of an ischemic stroke substantially decreased brain infarct volume and offered persistent advantages for the functional outcomes of patients compared to placebo. Based on these findings, it has been hypothesized that edaravone, a neuroprotective drug licensed in Japan, might protect skeletal muscle against I/R damage not only in an animal model but also in human settings. In addition, it has been observed that the use of additional antioxidants has therapeutic potential for I/R damage. Zhang et al. (2017) demonstrated that edaravone can significantly improve IR flap viability and protect flap vessels. Its effects are associated with scavenging oxygen free radicals, reducing the consumption of superoxide dismutase (SOD), reducing the magnitude of lipid peroxidation and inflammation, and protecting the functional structure of vessels in the initial reperfusion period (25).

The survival area was assessed 10 days postoperatively, following the work of Ballestin et al. (17). The entire size of the flap was 23.4 cm2 ($p > 0.05$). There were no statistically significant differences between the two groups. Moreover, edaravone prevents the invasion of inflammatory cells. The last common process of I/R damage involves edema, the recruitment of active leukocytes, and the development of membrane-attack complexes, all of which result in membrane rupture and cell death (26). Free radicals of oxygen start lipid peroxidation of cell membranes, leading to cellular malfunction and necrosis. The short half-life of free oxygen radicals makes it difficult to show their existence. Since the quantity of MDA (the end product of lipid peroxidation in cells) increases in proportion to the free oxygen radicals in I/R damage, we employed MDA testing to quantify reperfusion injury (13). However, there were no significant changes between the edaravone group and the control group in terms of MDA levels. Moreover, GSH is an intracellular antioxidant that neutralizes free radicals and oxygen-derived compounds (27). In this investigation, there were no significant differences in GSH levels between groups ($p > 0.05$). Finally, NO exerts antioxidant effects by decreasing leukocyte activation and increasing superoxide anion generation. In addition, reactive oxygen products reduce NO production (28). We found no statistically significant differences between the control and edaravone groups in our investigation in terms of NO levels.

By producing reactive oxygen species (ROS), proteases, and cationic peptides, active neutrophils may exacerbate I/R injury-induced host tissue damage. Neutrophils are activated by proinflammatory cytokines through nicotinamide adenine dinucleotide phosphate oxidase, resulting in the release of huge quantities of ROS (29). Neutrophils reduce tissue perfusion by obstructing capillaries, and an elevated neutrophil count is characteristic of I/R damage (30-32). The increased neutrophil counts in both the edaravone and control groups indicate that I/R injury was adversely affected. Using a modified Verhofstad grading system, (18) a histopathological investigation was conducted. In tissues with significant inflammation, inadequate blood supply, and severe reperfusion damage, increased edema, necrosis, and PMNL scores were found. However, while these measures showed higher values, there were no statistically significant changes between the two groups ($p > 0.05$). While vascular density was reduced, there was no significant difference between the edaravone and control groups ($p > 0.05$) (Fig. 2).

Although various studies have proven the impact of edaravone on I/R damage in flaps, as far as we know, this is the first complete study demonstrating its efficacy on the macroscopical, biochemical, and histological levels. The current investigation has some limitations. First, the sample size was small. Second, only the macroscopic necrotic area and histological and biochemical markers were assessed. Additional researches are required to validate the new therapeutic potential of edaravone for I/R injuries.

Conclusions

We know that edaravone is a valuable antioxidant and, as such, is likely to be effective against I/R injury. However, our findings did not show that edaravone has a favorable effect on I/R injury.

Author Contributions

Conception: O.A., Design: W.C.L, Supervision: O.A., F.Z., Resource: O.A., Materials: O.E., Data Collection and/or Processing: B.E.T., O.A., Analysis and/or Interpretation: Y.U., O.A., Literature Review: B.E.T., Writer: O.A., W.C.L., Critical Review: O.A., Y.U.

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