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Ethyl pyruvate improves skin flap survival after ischaemia reperfusion injury

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***Background & objectives*: Reperfusion after ischaemia is detrimental to the tissues. The oxidative stress created and cytokines released are mostly responsible in this process. In this study, ethyl pyruvate, a known agent for its anti-inflammatory and antioxidant properties, was used to investigate the effects on ischaemia/reperfusion injury on skin island flaps in rats.**

***Methods*: Sixty rats were randomly distributed in three groups (non-ischaemic, ischaemic and medication groups). Ethyl pyruvate was administered in the medication group with a dose of 50 mg/kg. After 24 h and one week, the animals were sacrificed, and the flaps were analyzed macroscopically, histopathologically, biochemically (total nitrite, malondialdehyde and myeloperoxidase).**

***Results*: Biochemical markers indicating oxidative stress, were found elevated in ischaemic group, whereas medication with ethyl pyruvate significantly reduced these values. There was a significant reduction ($P<0.05$) in the levels of these markers between ischaemic and medication groups. Ethyl pyruvate improved all the parameters significantly.**

***Interpretation & conclusion*: Ethyl pyruvate showed strong scavenger activity against reactive oxygen species. It could be a potential candidate to improve the flap viability in reconstructive microsurgery, especially in free tissue transfers. However, more studies are warranted in experimental models to confirm these findings.**

Key words Ethyl pyruvate - ischaemia - reperfusion injury - skin flap

Local and systemic consequences of ischaemia/reperfusion (I/R) injury may cause multiorgan failure, and even death. I/R injury is mediated mainly through toxic free radicals, named reactive oxygen species (ROS)[1]. Thrombosis, neutrophil infiltration, capillary narrowing, endothel dysfunction, release of cytokines and proinflammatory substances are triggered with reperfusion which yields ROS[1,2].

Pyruvate is capable of scavenging ROS; however, the instability of pyruvate in aqueous solutions makes it useless in practice[3]. To overcome this problem, Sims *et al*[4] introduced the ethyl ester of pyruvic acid

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known as ethyl pyruvate (EP). To circumvent the poor solubility of pyruvate, ethyl pyruvate was formulated in a calcium (Ca^{2+}) and potassium (K^{-}) containing solution named Ringer's ethyl pyruvate solution (REPS)[4]. Ethyl pyruvate has unique properties such as antithrombotic, anti-inflammatory and anti-cell death (apoptosis) effects that enable metabolic rescue for selected tissues[1,5-7]. Several studies have revealed the property of ethyl pyruvate as an antioxidant agent like pyruvate[8-10]. The main action of ethyl pyruvate as a ROS scavenger seems to come from its anti-inflammatory property[3].

The current study, we tried to ameliorate I/R injury on skin island flaps in rats with ethyl pyruvate, a novel anti-inflammatory and anti-ischaemic agent that has not been studied previously.

Material & Methods

This study was carried out in Animal Laboratory of Ankara Training and Research Hospital, Ankara, Turkey. EP was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and was used to prepare REPS containing 130 mM Na^{+}, 4 mM K^{-}, 2.7 mM Ca^{2+}, 130 mM Cl and 28 mM ethyl pyruvate. This study was approved by Animal Ethics Committee of Ankara Training and Research Hospital.

Sixty Wistar rats (*Rattus norvegicus*) weighing between 250 and 300 g were used in this study. They were housed at a constant temperature (24°C) under a 12 h:12 h light:dark cycle in separate cages with free access to food and water.

Ketamine HCl (40 mg/kg) and xylazine (5 mg/kg) were used to anaesthetize the animals. After appropriate shaving and cleaning, epigastric island flaps (4 cm×7 cm) were elevated ventrally as described by Petry and Wortham[11] (Fig. 1). Sixty rats were divided into three groups as group I (non-ischaemic group), group II (ischaemic group) and group III (medication group). Each group was further divided into two sub-groups, a and b. The animals were randomly distributed.

In the first group (n=20), the elevated flaps were re-adapted again without any maneuver. Half of the animals (n=10) in this group were sacrificed after 12 h (group Ia), while the rest (n=10) after seven days (group Ib). In group II, the pedicles of the flaps (superficial epigastric artery and vein) were clamped for 12 h to achieve global ischaemia which subsequently re-perfused. Half of the animals (n=10) in this group were sacrificed after 12 h of reperfusion (group IIa),

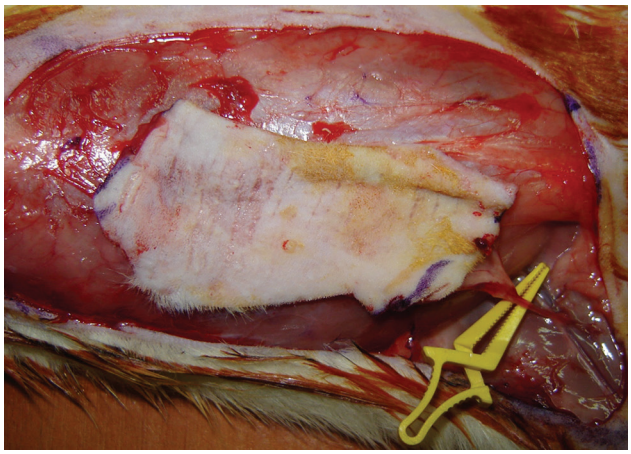

Fig. 1. The elevated flap with the pedicle (superficial epigastric artery and vein) just before it was clamped.

while the remaining half (n=10) were sacrificed seven days later (group IIb). The protocol of the third group was the same as group II, except 50 mg/kg REPS was administered intraperitoneally 30 min after the reperfusion[12,13]. In this group also 50 per cent (n=10) animals were sacrificed 12 h after the reperfusion (group IIIa), and the rest (n=10) were administered daily 50 mg/kg REPS intraperitoneally for seven days, and were sacrificed on day seven (group IIIb).

Biochemical analysis: Tissue samples obtained from the flaps (1 cm×4 cm) were stored in the liquid nitrogen. Total nitrite levels, malondialdehyde (MDA) levels and myeloperoxidase (MPO) activity were analyzed to measure the degree and quantity of reperfusion injury. Measurement of total nitrite levels[14] and MPO activity[15] was determined with spectrophotometry, and MDA levels were analyzed by the aid of thiobarbituric acid method[16,17].

Histopathological analysis: The specimens obtained from the flaps were stained with haematoxylin and eosin. Neutrophil infiltration, oedema, necrosis, neovascularization and fibrosis rates were assessed.

Analysis for necrosis: The flaps in groups Ib, IIb and IIIb were photographed, and the ratio of necrotic area to total flap area (4 cm×7 cm) was digitally analyzed with a software (AutoCAD, Autodesk Inc, San Rafael, CA, USA).

Statistical analysis: Distribution of data was controlled with Kolmogorov–Smirnov test. Comparison between groups was performed with Student's *t* test. The comparisons were done between groups Ia and IIa, IIa

and IIIa, Ib and IIb, IIb and IIIb. The statistical analyses were performed by SPSS, version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

All animals survived and no complications were noted in the study. The mean values of total nitrite and MDA levels and MPO activities were found elevated in the ischaemic groups (group IIa and IIb), whereas the medication groups (group IIIa and IIIb) demonstrated a significant drop (Table I).

Like biochemical markers, oedema, neutrophil infiltration and necrosis rates were significantly elevated in the ischaemic groups. Administration of ethyl pyruvate decreased these rates. Fibrosis, a chronic inflammatory finding was also observed less in the medication group compared to the ischaemic group. On the contrary, a significant neovascularization was obtained in the medication group when compared with the ischaemic group which demonstrated poor vascularization (Fig. 2). Table II indicates detailed information among the groups.

Table I. Mean and standard deviation values among the groups

Groups	Total nitrite (nmol/mg)	MDA (nmol/mg)	MPO (mU/mg)	Necrosis rate (%)
Group Ia (n=10)	0.11±0.09	0.31±0.17	21.61±7.70	
Group IIa (n=10)	0.29±0.11*	0.88±0.21*	80.61±29.70*	
Group IIIa (n=10)	0.19±0.05†	0.59±0.18†	22.31±4.51†	
Group Ib (n=10)	0.09±0.04	0.23±0.09	22.56±10.83	13.2±7.05
Group IIb (n=10)	0.58±0.25$^{\delta}$	0.66±0.22$^{\delta}$	170.3±53.48$^{\delta}$	43.2±16.12$^{\delta}$
Group IIIb (n=10)	0.28±0.09$^{\#}$	0.34±0.07$^{\#}$	39.54±18.14$^{\#}$	20.3±6.36$^{\#}$

$^{*}P<0.05$ compared to group Ia; $^{\dagger}P<0.05$ compared to group IIa; $^{\delta}P<0.05$ compared to group Ib; $^{\#}P<0.05$ compared to group IIb. Data presented as mean±SD (n=10). SD, standard deviation; MDA, malondialdehyde; MPO, myeloperoxidase

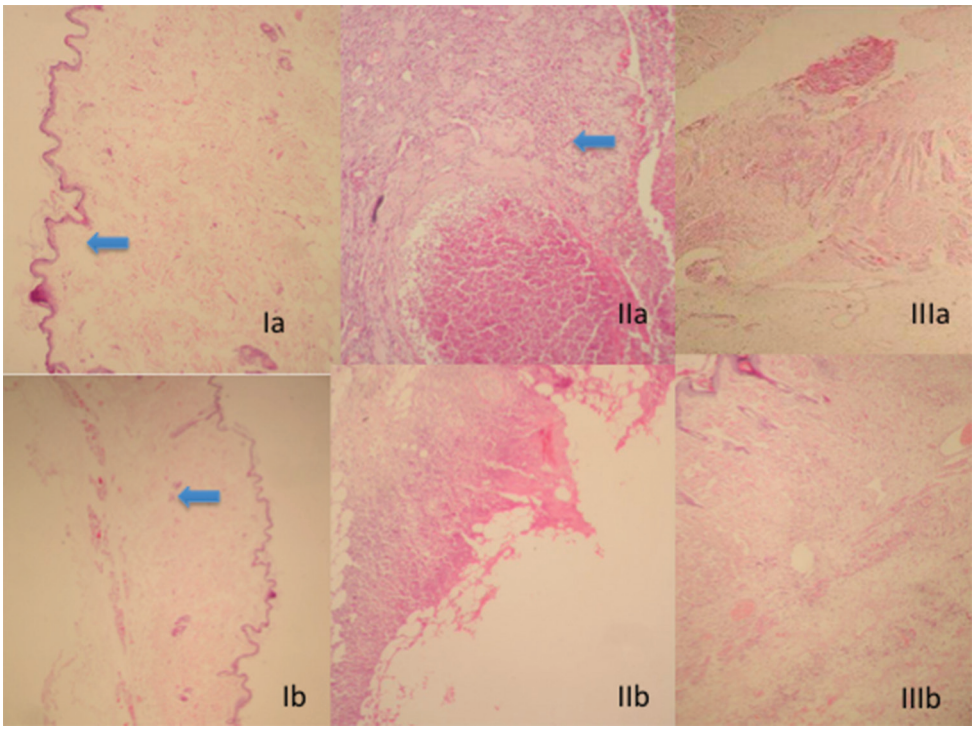

Fig. 2. Upper row represents the histopathologic slides showing oedema (arrow in Ia), neutrophil infiltration (arrow in IIa) and necrosis rates for the groups Ia, IIa and IIIa, whereas lower row shows slides for the groups Ib, IIb and IIIb. There is significant neovascularization in groups Ib (arrow in Ib) and IIIb. Fibrosis is lower in groups Ib and IIIb with regard to group IIb. Slides Ia, Ib, IIIa and IIIb, H&E staining ×10; IIa and IIb, H&E, ×20.

The necrotic area to the total flap area could be assessed macroscopically (Fig. 3). On 7th day, the necrotic ratio of the ischaemic group (group IIb) was almost half dimension of the elevated flap, whereas mean 80 per cent flap viability was observed in the medication groups (groups IIIa and IIIb). Table I demonstrates necrosis rates of the animals.

The biochemical markers (total nitrite, MDA and MPO) were found significantly elevated in the ischaemic groups when compared to the non-ischaemic groups (Table I). Medication with ethyl pyruvate diminished the oxidative stress and lowered the levels of measured markers which yielded significant concordance. Histopathological findings can be clearly seen among the groups regarding acute and chronic scores in Table II.

Discussion

Certain pyruvate esters such as ethyl pyruvate and methyl pyruvate have been evaluated previously and found more stable in aqueous solutions[4]. Hence, REPS was used in this study to see its effect on I/R injury in rats. Ethyl pyruvate was tested with several doses. Doses below 40 mg/kg have shown limited influences, whereas higher doses (above 50 mg/kg) had significant effects on I/R injury[3,12,18].

MPO has been used in various animal models of I/R injury as a marker of neutrophil infiltration[19]. MPO destructs the tissues enzymatically and generates oxidative stress. Like MPO, MDA, the end product of lipid peroxidation, is a good indicator of oxidative stress[20,21]. Nitrite and nitrate are the markers of nitric oxide (NO) synthesis[3]. NO is a well-known protector

Table II. Comparison of the histological scores among the three groups

Histological findings	Group Ia (non-ischaemic group)	Group IIa (ischaemic group)	Group IIIa (medication group)
Oedema	++	++++	++
Neutrophil infiltration	+	++++	+++
Necrosis rate	+	+++	++
	Group Ib	Group IIb	Groub IIIb
Oedema	++	++++	++
Neutrophil infiltration	+	++++	++
Necrosis rate	+	+++	+
Fibrosis	+	+++	++
Neovascularization	++++	+	+++

+, lowest; ++, lower; +++, medium; ++++, high

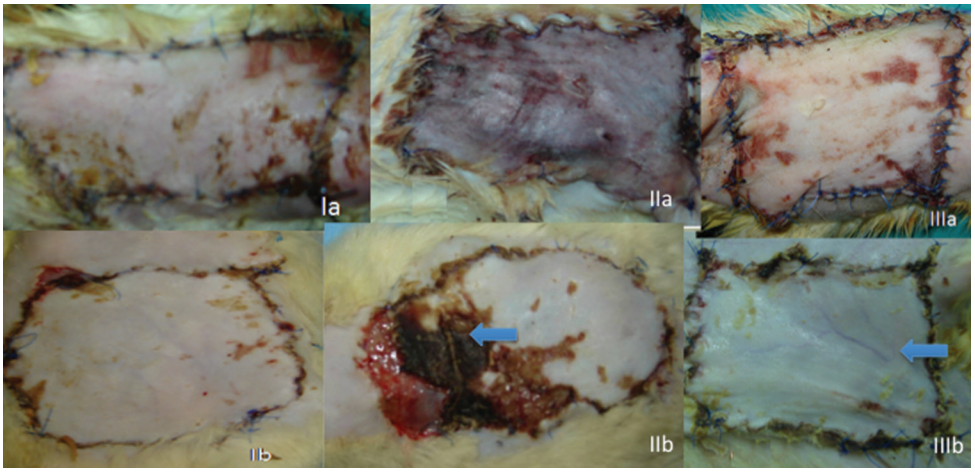

Fig. 3. Randomly selected flaps from different treatment groups showing medication with ethyl pyruvate improved the flap survival (panels IIIa,b). In the middle column, a sample from ischaemic group (panel IIb) indicates significant necrosis (arrow); on the other hand, arrow in panel IIIb shows 100 per cent survival rate.

against I/R injury[22]. Ischaemia causes NO release, yielding another ROS, peroxynitrite. Therefore, NO synthetase inhibitors are used in I/R injury.

Ethyl pyruvate has beneficial effects on thermal cutaneous injury in rats, mainly through cellular immune system[23]. It has been shown to improve the survival and alter systemic dysfunction[3]. Ethyl pyruvate significantly improved survival and outcome from shock in rat models[3,9]. The anti-inflammatory properties of ethyl pyruvate have had crucial effects on haemorrhagic shock. Ethyl pyruvate used as a resuscitation fluid was found superior in the survival of the animals in shock[24,25]. One of the critical consequences of shock is on the liver functions. Hepatic I/R injury is predominantly encountered during haemorrhagic shock, transplant surgery and trauma[26]. Shen *et al*[5] revealed that ethyl pyruvate had inhibitory effects through intrinsic mechanisms, on hepatic apoptosis and autophagy. The same mechanisms possibly act on brain tissue ensuring a reasonable protection against hypoxic brain injury[6]. According to this study, the neuroprotective effects of ethyl pyruvate were encountered with anti-apoptotic and anti-inflammatory actions.

A former study demonstrated that ethyl pyruvate not only prolonged the survival time of a rat model in septic shock but also increased interleukin (IL)-10 (anti-inflammatory cytokine) production and decreased a proinflammatory cytokine, IL-6[27]. In another study, the reduction of bacterial translocation and ROS production in intestine in thermal injury was significantly enabled with ethyl pyruvate[16]. Tsung *et al*[28] evaluated the effects of ethyl pyruvate against hepatic I/R injury in a rat model. Proinflammatory cytokines, both circulatory and hepatic, were significantly decreased in ethyl pyruvate treated animals. According to this study, extracellular signal regulations altered the process.

Many studies indicate that ethyl pyruvate is a potent agent against sepsis and septic shock[18,29,30]. According to these studies, ethyl pyruvate has beneficial effects on lung, kidney, intestine, pancreas and systemic haemodynamics; thus, multiorgan system dysfunction can be ameliorated with the use of ethyl pyruvate, successfully[18,31,32].

Coronary I/R injury and effects of ethyl pyruvate on cardiac function recovery have also been studied[33,34]. Myocardial infarct size and apoptosis after global cold ischaemia and reperfusion injury were reduced, whereas adenosine triphosphate levels were found increased and myocardial function after I/R injury was immediately improved with the administration of ethyl pyruvate.

In the current study, ethyl pyruvate was used for the attenuation of I/R injury in rat skin flap model. The biochemical markers were found increased in ischaemic group when compared to the non-ischaemic group. The medication group with ethyl pyruvate demonstrated a significant decrease in the levels of markers. Ethyl pyruvate lowered the contributing mediators in I/R injury. ROS-mediated actions were altered with the administration of ethyl pyruvate.

In conclusion, ethyl pyruvate showed a protective effect in I/R injury in rats; however, this was limited mainly to animal experiments. Additional clinical studies are warranted to see the effects of ethyl pyruvate in human experiments.

***Conflicts of Interest*:** None.

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