

# The protective effect of ethyl pyruvate on lung injury after burn in rats

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## ABSTRACT

**Objectives:** To investigate the effects of administered ethyl pyruvate (EP), a novel anti-inflammatory agent, on oxidoinflammatory and apoptotic pathways in the lung tissue of rats in a full-thickness burn model.

**Methods:** The study took place in Ankara Research and Training Hospital Animal Laboratory, Turkey in June 2006. Thirty-two rats were randomly divided into 4 groups in equal numbers as sham, burn, sham+EP, and burn+EP. The burn model, used produced a full thickness burn of the 30-35% of the total body surface area. Ethyl pyruvate was administered as 40 mg/kg intraperitoneally. Rats were sacrificed after 24 hours, acute lung injury (ALI) was evaluated by direct light microscopy and apoptosis was evaluated by caspase-3 staining. Oxidoinflammatory events were evaluated by determining the tissue levels of myeloperoxidase (MPO), lipid peroxidation products, and nitrite.

**Results:** No significant difference was observed in lung tissue nitrite and malondialdehyde levels among the study groups. Histopathological results revealed that ALI and apoptosis were significantly higher in the burn group and EP prevented this effect. Similar results were obtained in tissue MPO levels.

**Conclusion:** Ethyl pyruvate is a novel, potent anti-inflammatory agent. This agent prevented leukocyte infiltration, ALI, and apoptotic loss of the lung tissue in thermal injury.

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Despite the progress in the management of thermal injury, its complications, such as systemic inflammatory response syndrome, sepsis and multiple organ failure, continue to be the leading causes of mortality and morbidity.<sup>1,2</sup> These septic events often occur without an identifiable focus of infection. Gut hypoperfusion has been implicated as an initiating event in the development of septic complications and one of the common lethal complications has been acute respiratory distress syndrome (ARDS) and acute lung injury (ALI).<sup>3</sup> The pathophysiology of the lung insult without direct lung injury is complex and may be mainly caused by secondary damage from activated inflammatory cells by way of oxidoinflammatory cells. The role of reactive oxygen species (ROS) in lung injury in the septic states has been shown in a variety of animal models.<sup>4</sup> In sepsis-induced ARDS and ALI, the dependent lung regions are the sites of greatest inflammation and injury. There are some experimental studies suggesting that an apoptotic process triggered by oxidative stress results in lung injury.<sup>5,6</sup> There is evidence that apoptotic loss of parenchymal cells may have an association with organ dysfunctions, which seem to be one causes of mortality.<sup>7</sup> Ethyl pyruvate (EP) is a novel anti-inflammatory agent, which functions in cells as an endogenous antioxidant and free radical scavenger.<sup>8</sup> In the present study, we aimed to investigate the effects of administered EP on oxidoinflammatory and apoptotic pathways in the lung tissue of rats in a full-thickness burn model. To achieve this lung myeloperoxidase (MPO) content, lipid peroxidation products, and nitrite/nitrate levels were measured as indices of lung injury. Additionally, apoptosis and histopathology were evaluated in lung tissue.

**Methods.** The experiments described in this article were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. The experimental protocol was approved by the Ethical Committee of Ankara Numune Training and Research Hospital, and the study took place in Ankara Hospital Animal Laboratory, Turkey in June 2006. Thirty-two Wistar rats, weighing between 200 and 250 g were

used in this study. The rats were housed at constant temperature with a 12-hours period of light-dark exposure. Animals were allowed access to the standard rat chow and water ad libitum. The rats were randomly divided into 4 groups in equal number. The first group (sham group, group I, n=8) received sham burn and saline injection (1 mL/100 g). The second group (burn group, group II, n=8) received thermal injury and saline injection (1 mL/100 g). The third group (sham + EP group, group III, n=8) received sham burn and intraperitoneally EP injection (40 mg/kg) and the fourth group (burn + EP group, group IV, n=8) received thermal injury and intraperitoneally EP injection (same dose) 6 hours after thermal injury. The EP was purchased from Sigma-Aldrich Chemie GmbH, Riedstr. 2, D-89555 Steinheim, Germany. All animals were sacrificed 24 hours after thermal injury and both lungs were harvested through a midline sternotomy. To evaluate the burn-induced lung injury and apoptosis, tissue samples of lung were divided into 4 parts. One of them was fixed in 10% formaldehyde, and the other 3 parts were taken for biochemical assays. Lung specimens were kept frozen at -700°C until analysis. Animals were anesthetized with intramuscular injections of ketamine hydrochloride (Ketalar®, Eczacibasi, Turkey) (50 mg/kg) and xylazine (Rhompun®, Bayer, Turkey) (5 mg/kg). The backs of animals were shaved to allow direct skin contact between skin and hot water. A marked area of the shaved dorsal skin was exposed through a template and immersed in boiling water for 12 seconds. This procedure produced a full thickness burn of 30-35% of total body surface areas of rats.<sup>9</sup> The rats in sham group were exposed to room temperature water instead of boiling water. All animals in the group II and IV were resuscitated by intraperitoneal injection of 1 mL/100 g saline following burn injury and were allowed to feed with water and standard rat chow after recovering from anesthesia. No animals died within the first 24 hours of post-burn period. The MPO activity was determined by the method of Koike et al.<sup>10</sup> Lung tissue was homogenized in 20 mM potassium phosphate buffer (pH: 7.4) and the homogenate was centrifuged for 5 minutes at 10000 x g at 40°C. The supernatant was discarded and the pellet was resuspended in 50 mM potassium phosphate buffer (pH: 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The suspension was frozen and it was then sonicated for 10 seconds, incubated for 2 hours in a water bath (600°C), and centrifuged at 10000 x g for 5 minutes. The supernatants were used for MPO assay. The MPO activity was assessed by measuring the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of o-dianisidin. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 410 nm and 370°C. The level of malondialdehyde as an end product of lipid peroxidation determines lung tissue homogenized

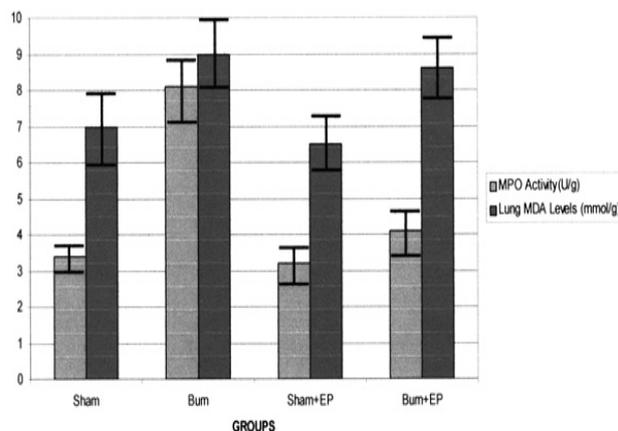
in the ratio of 1/10 (w/v) in 1.15% KCl solution by the aid of thiobarbituric acid method, and the results were obtained in nmol/g tissue weight.<sup>11</sup> Nitric oxide generation was estimated by a measurement of nitrite in lung tissue as follows: Tissue samples were homogenized in phosphate-buffered saline solution and centrifuged at 10000 x g for 20 minutes. Nitrite production was determined colorimetrically using Griess reaction in these supernatants.<sup>12</sup> The protein concentrations of the same supernatants were measured by the method of Lowry et al.<sup>13</sup> Nitrite levels were expressed in nmol nitrite/mg of protein. The specimens were fixed in 10% formalin for 24 hours, and standard dehydration and paraffin-wax embedding procedures were used. Hematoxylin and eosin-stained slides were prepared using the standard methods. Light microscopic analysis of lungs was performed by blinded observation to evaluate the pulmonary architecture, tissue edema formation, and infiltration of the inflammatory cells. The results were classified into 4 grades, where grade 1 represented normal histopathology; grade 2 indicated only limited neutrophil leukocyte infiltration; grade 3 represented moderate neutrophil leukocyte infiltration, perivascular edema formation, and partial destruction of pulmonary architecture; and finally grade 4 included dense neutrophil leukocyte infiltration, abscess formation, and complete destruction of pulmonary architecture.

In this study, we used immunohistochemical staining with caspase-3 to determine apoptosis in the lung tissue. Caspase-3 is a cysteine protease protein (CPP 32) that exists as inactive zymogen in all cells and it is involved in the development of apoptotic cell death in cell turnover. Paraffin sections of 5 mm were cut and deparaffinized. The antigen retrieval was performed by microwave pretreatment. The citrate buffer was preheated 4 times by incubation in a microwave oven at 750 W for 5 minutes. After cooling, slides were treated with hydrogen peroxide to block the endogenous peroxidase activity. Then, nonspecific binding of antibody was blocked by Ultra V block (Laboratory Vision/ Fremont, CA 94539) for 10 minutes, Caspase-3 antibody (CPP 32, Ab-4 dilution 1:200, Neomarkers Labvision, Fremont, CA 94539) was incubated in a moist chamber for 45 minutes followed sequentially with biotinylated goat anti-polivalent (Laboratory Vision) and streptavidin peroxidase complex (Laboratory Vision). After chromogen incubation, hematoxylin was used for nuclear counterstain. A case of chronic tonsillitis was included for positive control. Sections were examined by light microscopy. The numbers of positively stained cells in 5 high-power fields (x 400) were counted in the most intensively stained areas and their mean was calculated. All values are given as mean ± SD values. Statistical differences for values were evaluated using one-way analysis of variance followed by Tukey test.

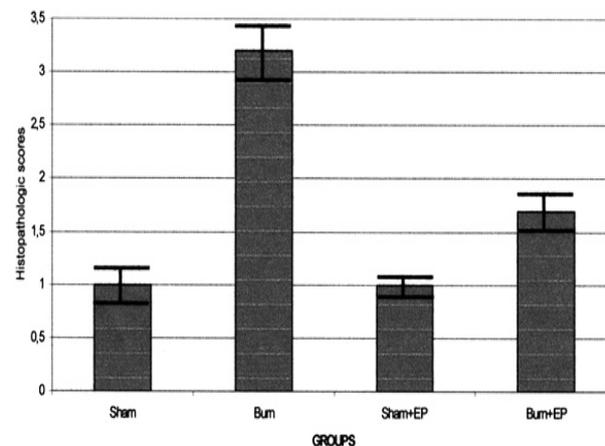
For all statistical test, we used the Statistical Package for Social Sciences version 10.0 software (SPSS Inc, Chicago, USA) and the  $p$  values less than 0.05 were considered significant.

**Results.** All animals in the groups survived the experiment period. The activity of MPO in lung tissue is demonstrated in **Figure 1**. In the burn group (group II), lung tissue MPO activity was found to be significantly increased ( $3.4 \pm 0.3$  versus  $8.1 \pm 0.7$ ) whereas EP caused a decrease in MPO activity ( $4.1 \pm 0.5$ ;  $p < 0.05$ ). Although MPO activity levels were still higher in the burn + EP group (group IV) when compared with the sham, this difference was not statistically significant. Although the malondialdehyde (MDA) levels of the burn group was higher than the sham group, the difference was not statistically significant (**Figure 1**). No significant difference was observed in lung tissue nitrite levels among the study groups. There were no significant light microscopic differences between lungs of sham and sham + EP group. In the burn group, interstitial edema with massive infiltration of the inflammatory cells was observed and the pulmonary architecture was severely damaged. In the burn + EP group, the pulmonary architecture was preserved and infiltration of inflammatory cells and edema decreased (**Figure 2**). In the sham and sham + EP groups, the caspase-3 staining resulted in 2-3 positive cells in 5 high-power fields. In the burn and burn + EP groups, the number of caspase-3 positive cells was found to be significantly increased. However in the burn + EP group, the number of the stained cells was reduced significantly compared with the burn group indicating a lower rate of apoptosis (**Figures 3, 4, & 5**).

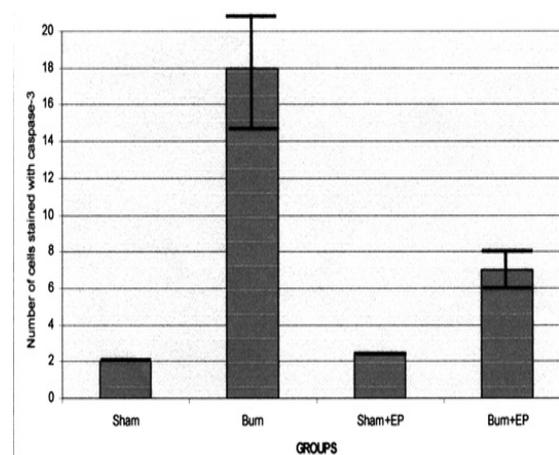
**Discussion.** In the present study, we demonstrated EP to reduce the number of apoptotic cells in the lung and to alleviate lung injury in burn model. Exposure of rats to thermal injury increased MPO activity in lung tissue, indicating the infiltration of polymorphonuclear neutrophils and the development of oxidative lung injury. Histopathological and immunohistochemical assessment also confirmed that the model of thermal injury used here substantially injured lung tissue, which was associated not only with necrosis but with apoptosis as well. Recently, it has been demonstrated that EP, a simple aliphatic ester derived from pyruvic acid, is an effective anti-inflammatory agent.<sup>8</sup> Sims et al<sup>14</sup> showed that, the pre- and post-treatment of rats with EP subjected to mesenteric ischemia-reperfusion tended to preserve normal intestinal mucosal histology, and significantly ameliorated the development of gut-mucosal hyperpermeability after reperfusion. Subsequently, Tawadrous et al<sup>15</sup> showed that resuscitating rats with EP instead of Ringer's lactate solution resulted in improved survival, decreased intestinal mucosal damage, and the



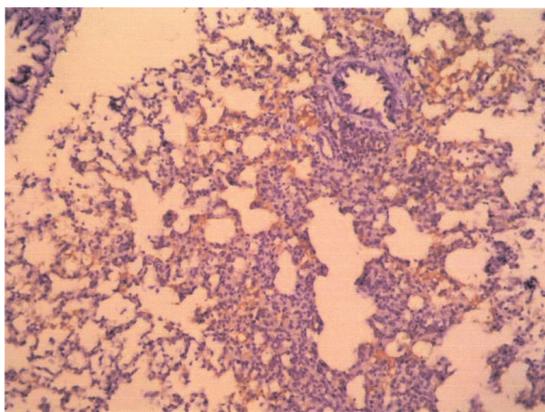
**Figure 1** - Lung myeloperoxidase (MPO) and malondialdehyde (MDA) levels. EP - ethyl pyruvate



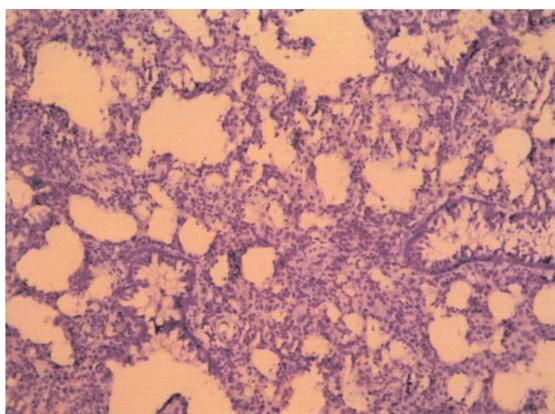
**Figure 2** - Histopathological scores of the lung tissue. EP - ethyl pyruvate



**Figure 3** - Number of positively stained cells with caspase-3. EP - ethyl pyruvate



**Figure 4** - Immunohistochemical staining for apoptosis in lung tissues by caspase-3 (x 200) in the burn group. Caspase-3 stained apoptotic cells and apoptotic bodies are seen.



**Figure 5** - Immunohistochemical staining for apoptosis in lung tissues by caspase-3 (x 200) in the burn + EP group.

amelioration of lipid peroxidation in the liver and gut. In view of the studies, Fink<sup>8</sup> proposed that some of the anti-inflammatory effects of EP could be attributed to its ability to function as a free oxygen scavenger and thereby block any ROS- dependent pathways leading to nuclear factor (NF)-KB activation. The ROS, generated by neutrophils, activates NF-KB, resulting in the excessive production of inflammatory cytokines, which may be the primary triggering agents for apoptosis in septic states. It was demonstrated experimentally that endotoxemia caused the activation of NF-KB in lungs through ROS-dependent mechanisms.<sup>16</sup> Clinically, NF-KB activation was also determined in patients with ALI.<sup>17</sup>

In our study, we found that EP decreased and elevates MPO activity, one of the markers of neutrophil accumulation in lung tissue, in the burn group. Also, it was documented with hematoxylin-eosin staining; lung histology was preserved by EP after thermal injury. Most interestingly, the EP treatment significantly attenuated

the burn induced apoptosis, which was documented by caspase-3 staining immunohistochemically. No significant difference was observed in lung tissue nitrite and MDA levels among the study groups. This result may be due to early sacrifice of the rats after burn.

In conclusion, the use of EP inhibited MPO activity; which resulted in a reduction of apoptosis in the lung in the burn model. We believe that the use of EP deserves additional investigation for its possible antiapoptotic potential in septic states besides its well-known antioxidant and anti-inflammatory effects.

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