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Protective effect of trapidil against oxidative organ damage in burn injury

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Abstract

Animal models of thermal injury indicate reactive oxygen species and inflammatory cytokines as causative agents in tissue injury on various organs distant from the original wound. Trapidil has various properties, such as inhibition of platelet aggregation and lipid peroxidation as well as reduction of the inflammatory response to injury. This study was designed to determine the possible protective effect of trapidil treatment against oxidative organ damage in lung, intestine and kidney induced by cutaneous thermal injury.

Thirty Wistar rats were randomly divided into five groups. Sham group ($n = 6$) was exposed to 21 °C water while burn-3 h group ($n = 6$) and burn + trap-3 h group ($n = 6$), burn-24 h ($n = 6$) and burn + trap-24 h groups were exposed to boiling water for 12 s to produce a full thickness burn in 35–40% of total body surface area. In both burn + trap-3 h and burn–trap-24 h group, 8 mg/kg trapidil was given intravenously immediately after thermal injury. Three and 24 h later, tissue samples were taken for biochemical analysis from lung, intestine and kidney and blood samples were obtained to determinate serum TNF-α levels. Cutaneous thermal injury caused a significant increase in myeloperoxidase (MPO) activity and malondialdehyde (MDA) and 3-nitrotyrozine (3-NT) levels in all tissues and elevated serum TNF-α levels at post-burn 3 and 24 h. Trapidil treatment significantly reduced in biochemical parameters, as well as serum TNF-α levels. These data suggest that trapidil has a protective effect against oxidative organ damage in burn injury.

Keywords: Burn injury; Trapidil; Nitric oxide; TNF-α; Lipid peroxidation; 3-Nitrotyrosine

1. Introduction

Despite recent advances in the management of burn care, thermal trauma may cause damage to multiple organs distant from the original burn wound and may lead to multiorgan failure. Thermal injury is accompanied by complex events that exert deleterious effects on various organs in different systems distant from the original burn wound. Although the pathophysiological mechanisms of tissue injury remains unclear, there is increasing evidence that both oxidative and nitrosative stress have an important role in the development of multiorgan failure after thermal injury [1,2].

After thermal trauma, all tissues are subjected to ischemia and consequently, especially during burn resuscitation, reperfusion injury occurs [3,4]. Recently, several studies suggested that oxidative and nitrosative stress initiate an inflammatory cascade that includes acute phase protein synthesis, upregulation of inflammatory adhesion molecules and pro-inflammatory cytokine, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), release in ischemia reperfusion injury and burn injury [3,5,6]. Activated inflammatory cascade causes local and systemic neutrophil sequestration, which is source of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Thus, tissue or organ injury after thermal trauma appears to be mediated by both ROS and RNS, such as hydroxyl radical, superoxide anion, hydrogen peroxide, nitric oxide (NO) and peroxyni-

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trite ($ONOO^-$) [7]. Several studies have demonstrated that infiltrated neutrophils lead to formation of ROS, thereby contributing to organ injury distant from the original burn wound [4,8]. It has been shown that burn injury, associated with lipid peroxidation, mediated by ROS, is believed to be an important cause of oxidative damage to cellular membranes, and eventually cell death [9]. Furthermore, NO has been implicated in the pathogenesis of inflammatory processes including burn injury [2], and it participates in the regulation of microcirculation in several systems in both physiologic and pathological settings [10].

Trapidil which is clinically used as an antianginal drug, is a phosphodiesterase and platelet-derived growth factor inhibitor [11]. The pharmacological properties of trapidil include nitroglycerine-like vasodilatation, inhibition of platelet aggregation via thromboxane A_2 (TxA_2) inhibition, facilitation of the biosynthesis of prostacyclin and reduction of lipid peroxidation [12,13]. Moreover, trapidil inhibits secretion of interleukin-6 (IL-6) and interleukin-12 (IL-12) and suppresses production of TNF-α [14].

Recently, it has been demonstrated that trapidil has protective effects of different experimental I/R and inflammatory models [15–17]. However, it appears that there is no published data about the effects of trapidil on different systems after thermal injury. For this reason, in this study, we aimed to investigate whether trapidil had any beneficial effects on burn-induced organ damage in rat. Furthermore, we have also investigated the relationship between trapidil and peroxynitrite pathway in present study.

2. Materials and methods

2.1. Animals

The experimental procedures performed in this study were in concordance with the guidelines of Turkish National Institutes of Health. The experimental protocol was approved by the Ethical Committee of Mersin University. Wistar rats, weighing between 200 and 250 g, were used in this study. The rats were housed at constant temperature with 12-h periods of light–dark exposure. Animals were allowed to access to standard rat chow and water ad libitum. A 1-week period of acclimatization was used in this study.

2.2. Experimental design

After stabilization period, 30 rats were randomly divided into five groups. The first group (sham group, $n = 6$) was exposed to 21 °C water and vehicle (saline, 1 ml/100 g) was administered intravenously (i.v.). The second group (burn-3 h group, $n = 6$) was exposed to thermal injury and was given vehicle i.v. immediately after burn. The third group (burn + trapidil-3 h group, $n = 6$) received thermal injury and 8 mg/kg trapidil (Rocornal UCB, Germany) was administered intravenously immediately after the burn. Group IV (burn-24 h group) was exposed to thermal injury and vehicle was administered i.v. immediately after burn. Fifth group (burn + trapidil-24 h group) was exposed to thermal injury and trapidil was administered i.v. immediately after burn. In groups IV and V, the injections of trapidil or vehicle were repeated at 12 h following burn injury. All animals in groups II and III were sacrificed 3 h after thermal injury whereas the animals in groups IV and V were sacrificed 24 h after burn injury. A midline laparotomy was performed and the samples of ileum, kidney and lung were harvested for biochemical evaluation. Then, the blood samples were obtained for serological and biochemical analysis by cardiac puncture.

2.3. Thermal injury

Animals were anaesthetized with intramuscular injections of ketamine hydrochloride (50 mg/kg) and morphine sulphate (15 mg/kg). The burn model described by Walker et al. was used in this study [18]. The backs of animals were shaved to allow direct skin contact between skin and hot water. Next, a corresponding metal template immersed in boiling water for 12 s was applied to produce a full thickness burn. The total area of the burn was 30–35% of total body surface of the rat. The rats in sham group were exposed in identical setting where room temperature water (21 °C) was used instead of boiling water. All animals in groups II, III, IV and V were resuscitated with intraperitoneally injection of 2 ml/100 g saline following burn injury and were allowed to feed ad libitum with water and standard rat chow after recovering from anaesthesia.

2.4. Detection of MDA levels

Tissues were homogenized in 0.15 mM KCI for malondialdehyde (MDA) determination. After the homogenate had been centrifuged at 3000 rpm, the MDA levels in tissue homogenate supernatant, and plasma were determined by thiobarbituric acid (TBA) reaction according to Hiroshi and Yagi. The principle of the method depends on the colorimetric measurement of the intensity of the pink colour produced by the interaction of the barbituric acid with MDA. The colour reaction with 1,1,3,3 tetraethoxypropane was used as the primary standard [19].

2.5. Measurement of tissue myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a haem-containing enzyme within the azurophil granules of neutrophils; therefore. measurement of MPO activity was used as a simple quantitative method for detecting leukosequestration. A tissue specimen of 300 mg was homogenized in 0.02 M EDTA (pH 4.7) in a Teflon Potter homogenizer. Homogenates were centrifuged at 20,000 × g for 15 min at +40 °C. After pellet was re-homogenized in 1.5 ml 0.5%

hexadecyltirimethylammonium bromide (HETAAB) prepared in 0.05 M KPO_4 (pH 6) buffer, it was re-centrifuged at 20,000 × *g* for 15 min at +40 °C. The determination of lung tissue and serum MPO activity depends on the fact that it reduces *o*-dianozidine; hence, reduced *o*-dianozidine was measured at 410 nm by spectrophotometer [20].

2.6. 3-Nitrotyrosine detection

3-NT was obtained from Sigma Chemical Co. (St. Louis, MO, USA) H_2O_2, sodium acetate, citrate, NaOH, MnO_2, H_3PO_4, KH_2PO_4 and K_2HPO_4 were purchased from Merck Chemical Co. (Deisenhofen, Germany) for measurement of 3 NT/total tyrosine. All organic solvents were HPLC grade. The tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4). Equivalent amounts of each sample were hydrolyzed in 6N HCI at 100 °C for 18–24 h, and then samples were analyzed on a HP 1049 HPLC apparatus. The analytical column was a 5 μm pore size Spherisorb ODS-2 C18 reverse-phase column (4.6–250 mm; Alltech, Deerfield, IL, USA). Guard column was a C18 cartridge (Alltech). The mobile phase was 50 mmol/I sodium acetate/50 mmol/I citrate/8% methanol, pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml min^{-1} and UV detector set 274 nm. 3-NT peaks were determined according to its retention time and the peaks were either confirmed by spiking with added exogenous 3-NT (10 μmol/I) reducing to aminotyrosine [21].

2.7. Analysis of serum TNF-α levels

Serum samples were stored at 70 °C until used for assay. TNF-α levels in the serum samples were measured by enzyme-linked immunosorbent assay (ELISA) method. Specific TNF-α ELISA kits (Bio Source, USA, lot no: 042904) were then used for the serum TNF-α assays.

2.8. Statistical analysis

Statistical evaluation for proportional comparisons for biochemical values and serum TNF-α were analyzed using one-way ANOVA followed by a Student–Neuman–Keuls test. All data were presented mean ± S.D. for the results. *p*-values less than 0.05 were considered as statistically significant. In all computations, SPSS (ver. 9.05) statistical package programme was used.

3. Results

3.1. Myeloperoxidase (MPO) activity

In both 3 and 24-h burn groups, tissue MPO activity significantly increased in the lung, kidney and ileum after thermal injury, when compared to sham group ($p < 0.0001$, $p < 0.005$). Trapidil treatment significantly reversed the

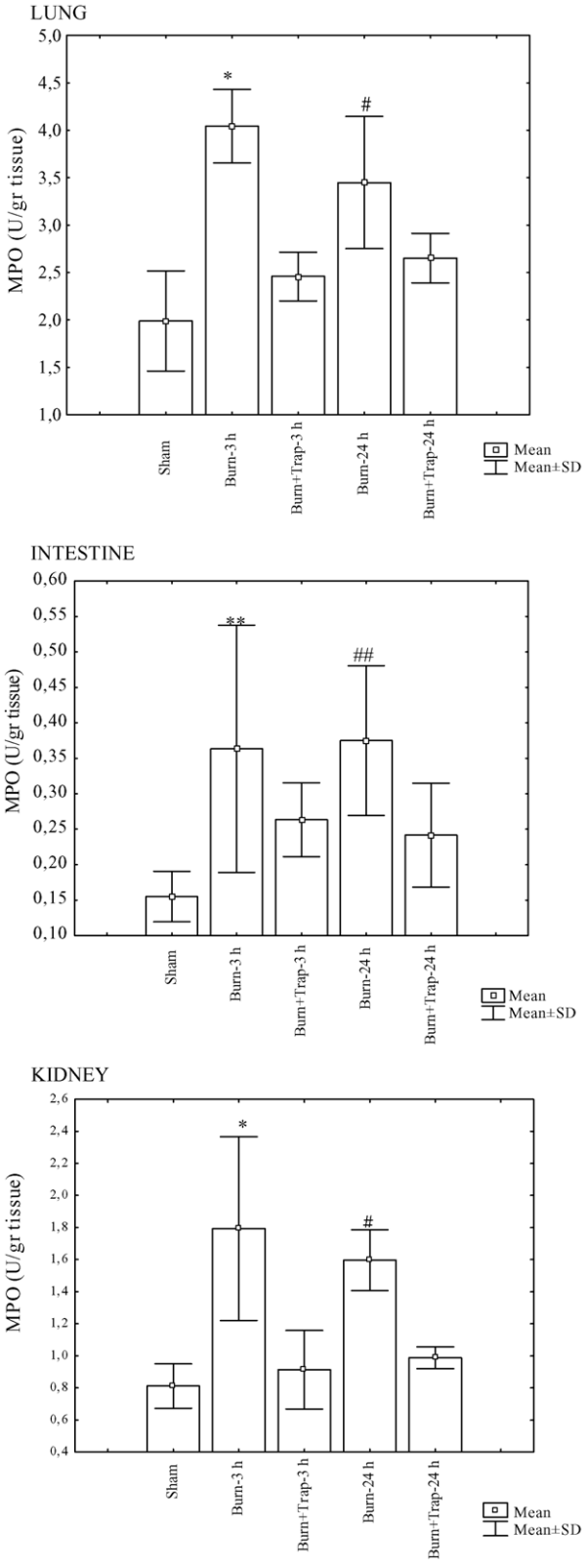
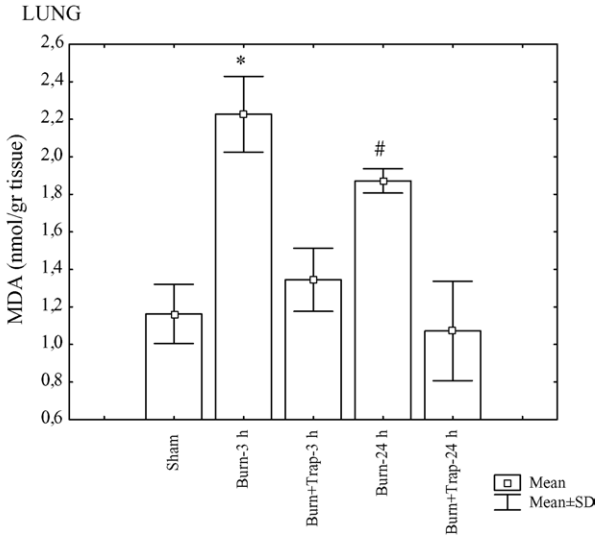
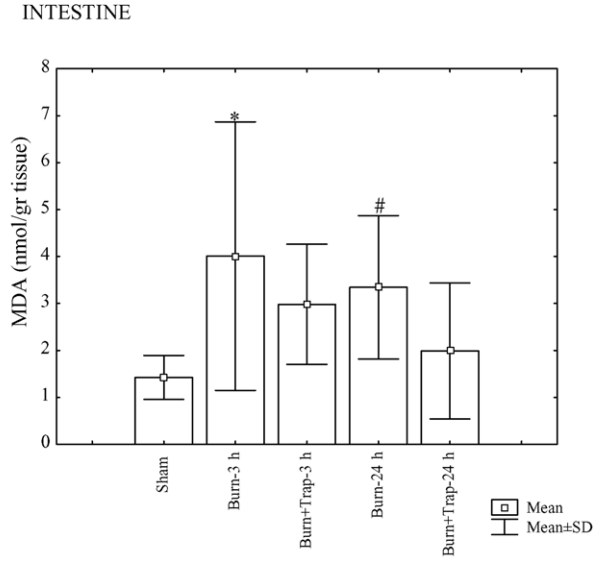


Fig. 1. MPO activities in lung, intestine and kidney in all groups. Data expressed mean ± S.D.; $^{*}p < 0.0001$ and $^{**}p < 0.005$, compared with sham and burn + trap-3 h groups; $^{\#}p < 0.0001$ and $^{\#\#}p < 0.005$, compared with sham and burn + trap-24 h groups.





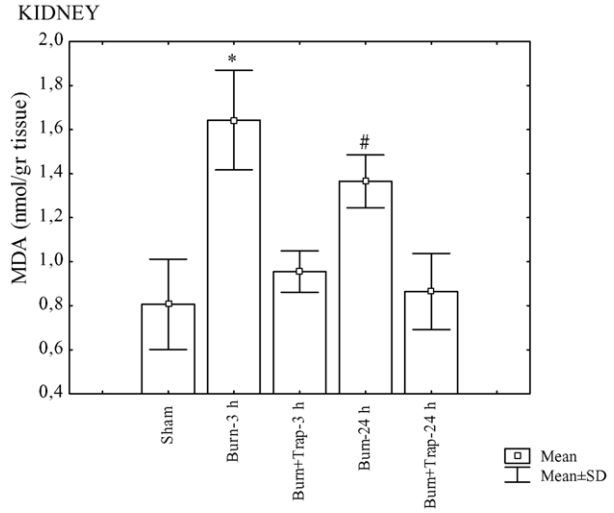
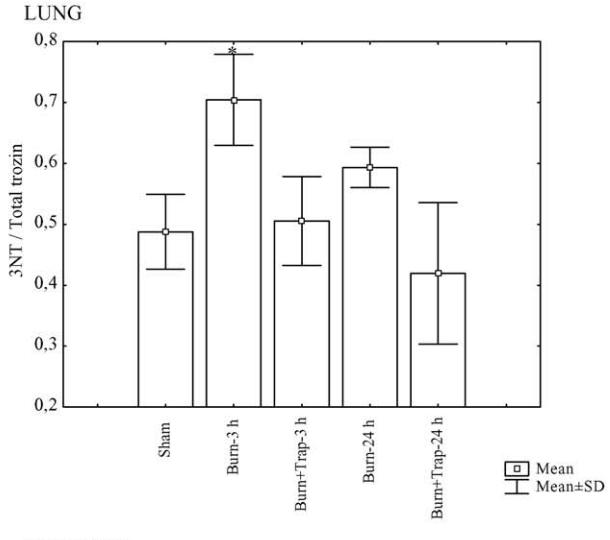
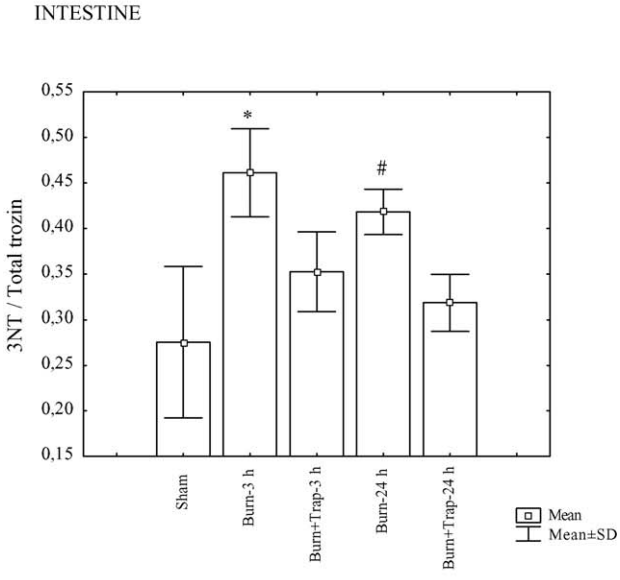


Fig. 2. MDA levels in lung, intestine and kidney in all groups. All data presented mean ± S.D.; $^{*}p < 0.0001$, compared with sham and burn + trap-3 h groups; $^{\#}p < 0.0001$, compared with sham and burn + trap-24 h groups.





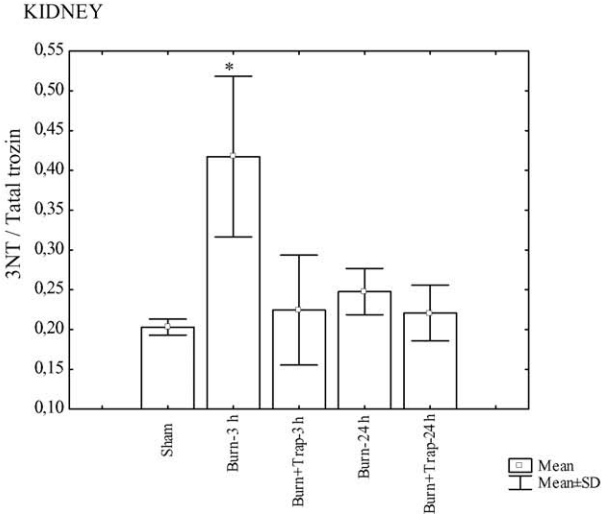


Fig. 3. 3-NT levels in lung, intesine and kidney in all groups. Data expressed mean ± S.D.; $^{*}p < 0.0001$, compared with sham and burn + trap-3 h groups; $^{\#}p < 0.0001$, compared with sham and burn + trap-24 h groups in intestine only.

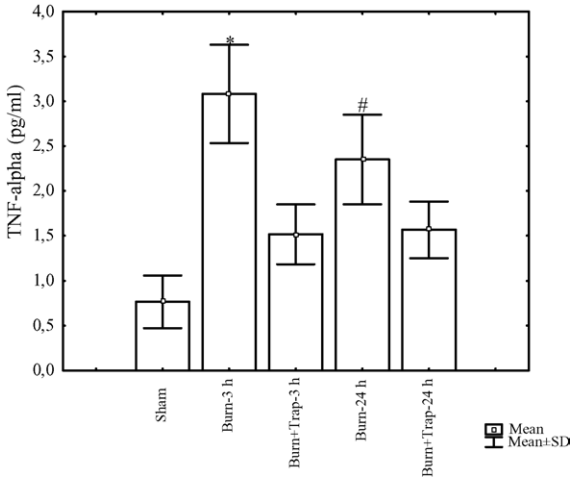


Fig. 4. Serum TNF-α levels in all groups. Data expressed mean ± S.D.; $^{*}p < 0.05$, compared with sham and burn + trap-3 h groups; $^{\#}p < 0.05$, compared with sham and burn + trap-24 h groups.

elevations in MPO activity in all tissues at both time points after thermal injury ($p < 0.05$) (Fig. 1).

3.2. Malondialdehyde (MDA) levels

MDA levels of tissues correlated with MPO activity. In both burn-3 h and burn-24 h groups, MDA levels of lung, kidney and ileal tissue were found to be significantly increased than those of the sham group ($p < 0.0001$). The levels of MDA depressed with trapidil treatment in all tissues ($p < 0.0001$) (Fig. 2).

3.3. 3-Nitrotyrosine (3-NT) levels

3-NT levels in tissues are demonstrated in Fig. 3. Thermal injury caused significantly an increase of 3-NT levels in the lung, kidney and ileum in the burn-3 h group when compared to the sham group ($p < 0.0001$). In 24-h burn group, burn injury led to a significant rise in 3-NT in only ileal tissue ($p < 0.0001$). Trapidil treatment significantly reversed the elevations in 3-NT levels of all tissues in the burn + trapdil-3 h group, and depressed the ileal 3-NT level in the burn + trapidil-24 h group ($p < 0.0001$) (Fig. 3).

3.4. TNF-α values

Burn injury significantly increased serum TNF-α levels in both 3 and 24 h groups. This results were significantly higher when compared sham group ($p < 0.05$). Trapidil treatment inhibited TNF-α synthesis in both burn + trapidil-3 h and burn + trapidil-24 h groups ($p < 0.05$) (Fig. 4).

4. Discussion

The results in this study demonstrated that trapidil treatment immediately after thermal injury reduces the post-burn oxidative and nitrosative organ damage, as evidenced by changes in MDA and 3-NT levels and MPO activity and suppressed TNF-α production. These findings suggest that trapidil has a protective effect in the burn-induced oxidative and nitrosative tissue injury, which may be attributed to its anti-inflammatory and antioxidant properties.

Thermal injury of the skin results in both local tissue damage and a systemic inflammatory response which is extremely complex, resulting local burn tissue injury, as well as systemic effects involving organs distant from the burn area itself [1,3]. A local burn injury activates several pro-inflammatory cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor (TNF-α) resulting in generalized neutrophil sequestration and a priming of local and systemic neutrophils and macrophages [3,5]. Activated neutrophils are a potential source of toxic oxygen and nitrogen products, which further cause tissue damage. Zhou et al. have clearly revealed that trapidil inhibits the monocytic CD40 pathway, thereby reducing the production of TNF-α, IL-6 and IL-12 [14]. In our study, serum TNF-α levels significantly elevated after thermal injury. TNF-α production was inhibited by trapidil treatment in both treated groups (Fig. 4). These data indicate that trapidil has a potent inhibitor effect on TNF-α production in burn injury. We believe that this effect of trapidil may contribute at reducing of tissue damage after thermal injury.

Thermal cutaneous injury leads to a transient splanchnic vasoconstriction, which causes the damage of the intestine and kidney due to ischemia reperfusion (I/R) injury [22,23]. Reperfusion is typically associated with neutrophil accumulation both local and systemic. Generalized tissue inflammation is present in injured organs within hours of injury, even in the absence of shock. It has also been demonstrated that intravascular haemolysis in burn injury, as well as the onset of lung injury after thermal trauma of the skin were prevented by neutrophil depletion in animal studies, suggesting that neutrophils play an important role in the development of remote organ injury [9,24]. Several studies showed that neutrophil accumulation in liver, lung, kidney and gastric mucosa might be involved in the pathogenesis of burn injury in these distant organs [1,8,23]. In our study, tissue MPO activity, primarily found in neutrophils and levels of MPO in tissues reflects neutrophil sequestration, significantly increased in lung, intestine and kidney tissues at 3 and 24 h after burn injury. Trapidil treatment inhibited neutrophils infiltration in these organs as evidenced by reduced MPO activity in treated groups (Fig. 1). There are no evident findings for explanation of inhibiting effect of trapidil in English literature. However, the relationship between trapidil and platelet-derived growth factor (PDGF), which has chemotactic capacity for macrophages and neutrophils and strongly inhibited by trapidil, may plays a pivotal role in this effect [11,25,26].

Experimental and clinical studies have shown that a local burn insult produces oxidant-induced organ changes as evidenced by increased lipid peroxidation in different

organs, such as intestine, liver, lung and kidney [22,23]. Lipid peroxidation, mediated by ROS, is believed to be an important cause of oxidative damage to cellular membranes, and eventually cell death in burn injury [4]. However, also RNS, such as NO and $ONOO^-$ play an important role in lipid peroxidation after thermal injury [12]. $ONOO^-$ directly causes lipid peroxidation on many cellular membranes, thereby disturbing their function [26].

The release of free arachidonic acid, the precursor of prostaglandins and TxA_2, activates phospholipases, thus leading to increased prostanoid production, free radical release and lipid peroxidation [27]. In our study, we observed that the levels of tissue MDA, which is the product of lipid peroxidation, were significantly increased in lung, intestine and kidney tissues at both 3 and 24 h after thermal injury. In contrast, trapidil treatment inhibited MDA elevations significantly and reversed back to control levels. This set of data showed that lipid peroxidation was prevented with trapidil treatment. Thus, based on these findings, the protective effect of trapidil in burn-induced organ damage, in part, may be attributed to the membrane-stabilizing effect of trapidil by the inhibition of TxA_2 synthesis and in part, trapidil may also be antagonised by the cytotoxicity of peroxynitrite.

Recently, it has been demonstrated that NO plays a crucial role of the cellular injury in burn. Burn trauma produces a significant NO overproduction. The toxicity of NO has been attributed to the $ONOO^-$, which is formed by reaction of NO with superoxide anion and called as the ugly face of NO [28]. NO and $ONOO^-$ react with cellular lipid and protein components, consequently disturbing their function [3]. Another pathway of cell death, triggered by peroxynitrite, leads to the loss of cellular energetic via the induction of DNA single strand breaks, and subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS) [29]. PARS activation depletes the intracellular concentration of energetic substrate, nicotinamide dinucleotide (NAD), thus leading to cellular dysfunction and cell death [6].

The nitration of protein tyrosine residues by $ONOO^-$ results in the formation of 3-NT, the foot print of $ONOO^-$ and indirectly NO, and the appearance of this product in tissue samples is taken as a diagnostic tool for exposure to $ONOO^-$ and NO [30]. Several studies have reported that increased level of 3-NT has been detected in rat intestinal mucosa following thermal injury [31,32]. In present study, we observed that the levels of 3-NT were increased in lung and kidney tissues at 3 h burn group and in intestine at both 3 and 24 h burn groups and trapidil treatment significantly reversed these increases (Fig. 3). Thus, it seems that trapidil may be inhibited $ONOO^-$ formation or neutralised peroxynitrite-mediated cellular injury in burn.

Additionally, adhesion molecules, such as P-selectin, which mediates rolling of leukocytes along the endothelium and intercellular adhesion molecule-1 (ICAM-1), which mediates binding of leukocytes on the endothelium during the reperfusion, are normally expressed at basal level, but their expressions can be enhanced by several cytokines in various inflammatory processes including IR injury and burn trauma [33,34]. It has been shown that $ONOO^-$ enhances expression of P-selectin and ICAM-1 in human endothelial cells [35]. Furthermore, the use of TxA_2 inhibitors, such as trapidil, was demonstrated to attenuate diapedesis and ICAM-1 expression in I/R model [36]. In our study, although we did not study these adhesion molecules, we think that trapidil treatment might also have reduced the expression of P-selectin and upregulation of ICAM-1 in tissues in this experimental study.

In summary, thermal injury induces oxidative and nitrosative organ damage distant from original wound. Burn-induced tissue injury in lung, intestine and kidney are dependent upon the formation of oxygen and nitrogen radicals. Treatment with trapidil reduced the burn-induced organ damage by multiple effects on the lipid peroxidation, peroxynitrite pathway and inflammatory cytokines such as TNF-α.

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