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The effect of *N*-acetylcysteine on oxidative stress in intestine and bacterial translocation after thermal injury

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Abstract

Ischemia due to transient splanchnic vasoconstriction following major burns causes oxidative and/or nitrosative damage in intestinal tissue followed by reperfusion injury. Thus, burn injury leads to breakdown in the intestinal mucosal barrier which can induce bacterial translocation (BT). As an antioxidant and anti-inflammatory agent the protective effects of *N*-acetylcysteine (NAC) are documented in several studies. This study was designed to determine the effect of NAC treatment on the oxidative stress in the intestine and BT after burn injury. To evaluate this, 32 Wistar rats were randomly divided into four groups as sham ($n = 8$), burn ($n = 8$), pre-burn, NAC injection (150 mg kg^{-1}, intraperitoneally) 15 min before thermal injury ($n = 8$), post-burn, NAC injection (150 mg kg^{-1}, intraperitoneally) 2 h after thermal injury. Under anesthesia, the shaved dorsal skin of rats was exposed to boiling water for 12 s to induce burn injury in a standardized manner. Twenty-four hours later, tissue samples from mesenteric lymph nodes (MLN), spleen, and liver were obtained under sterile conditions for microbiological analysis and ileum samples were harvested for biochemical analysis. In the burn group, the incidence of isolating bacteria in MLN, spleen, and liver specimens was significantly higher than other groups. NAC treatment prevented burn-induced BT in both pre- and post-burn groups. Thermal injury caused a significant decrease in glutathione (GSH) level, significant increases in malondialdehyde (MDA) and myeloperoxidase (MPO) activity at post-burn 24th hour. Treatment of rats with NAC significantly elevated the reduced GSH levels while decreasing MDA levels and MPO activity. These data suggested that NAC has a crucial cytoprotective role in intestinal mucosal barrier and preventive effects against burn injury-induced BT.
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Keywords: *N*-Acetylcysteine; Bacterial translocation; Thermal injury

1. Introduction

Despite recent advances in the management of burn care systemic inflammatory response syndrome (SIRS), sepsis, and multiple organ failure (MOF) still continue to be a leading cause of mortality and morbidity [1,2]. These septic events are often manifest in the absence of an identifiable focus of infection. Gut hypoperfusion has been implicated as an initiating event in the development of septic complications and gastrointestinal tract has been defined as the "motor of MOF" [3,4]. Normally, in the gut, there is homeostasis between the intraluminal bacteria, their product and intestinal mucosal barrier. Thermal cutaneous injury leads to a transient and selective splanchnic vasoconstriction which is associated with decreased mesenteric blood flow and damage of the mucosal barrier due to ischemia reperfusion (I/R) injury. This phenomena promotes bacterial translocation (BT) from the gut [5,6]. The underlying mechanisms in the pathopyhsiology of the mucosal barrier damage is attributed to ischemia reperfusion injury. I/R injury to small intestine causes local production of the reactive oxygen species (ROS), such as hydroxyl radical, superoxide anion, hydrogen peroxide and reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite are thought to play a pivotal role in gut epithelial damage [7]. This in turn, creates a breakdown in the intestinal mucosal barrier

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and causes the distruption of mucosal integrity. This event may facilitate BT and release of endotoxins leading to septic complications and MOF in burn patients [8].

N-Acetylcysteine (NAC) is one of the most widely investigated pharmacological agent among free radical scavengers. NAC, which has a thiol group, is a well-known precursor of glutathione. It reacts best with the hydroxyl radical and hypochlorous acid and is poorly reactive with hydrogen peroxide and the superoxide radical [9]. Exogenously administrated NAC protects against I/R-induced tissue damage due to its ability to scavenge ROS [10]. Furthermore, NAC also elicits beneficial effects on inflammation process, such as suppression of cytokine expression/release, inhibition of adhesion molecule expression, and inhibition of nuclear factor kappa B (NF-κB) [11–13]. Although, experimental studies have shown protective effects of NAC on hepatic, renal, and intestinal I/R injury [14,15,10] and lung injury in sepsis models [16], there are no reports in literature about the effect of NAC on the small intestine after thermal injury. The aim of this study is to investigate the effect of NAC on intestinal oxidant damage and BT in burn injury.

2. Materials and methods

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. Thirty-two Wistar rats, weighing between 200 and 250 g were used in this study. The rats were housed at constant temperature with 12-h period of light–dark exposure. Animals were allowed to access to standard rat chow and water ad libitum. A 1-week period of acclimatization was use in this study.

2.1. Experimental design

The rats were randomly divided into four groups in equal number. The first group (sham group, group I, $n = 8$) received sham burn and saline injection (1 mL kg^{-1}). The second group (burn group, group II, $n = 8$), received thermal injury and saline injection (1 mL kg^{-1}). The third group (pre-burn group, group III, $n = 8$), received thermal injury and NAC injection (150 mg kg^{-1}, intraperitoneally) 15 min before thermal injury. The fourth group (post-burn group, group IV, $n = 8$), received thermal injury and NAC injection (same dose) 2 h after thermal injury.

2.2. Thermal injury

Animals were anesthetized by intramuscular injection of ketamine hydrochloride (50 mg/ kg), and xylazine (5 mg/ kg). The burn model described by Walker et al. was used in this study [17]. The backs of animals were shaved to allow direct skin contact between skin and hot water. Next, a corresponding meta 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 sham control animals were exposed to room temperature water in an identical setting. All animals in group II and group III were resuscitated with 2 ml/100 g saline intraperitoneally following burn injury. After recovering from anesthesia, all animals were allowed to access to water and standart rat chow. No animals died with in the first 24 h post-burn period.

2.3. Tissue sampling

All animals were scarified 24 h after the thermal injury. To evaluate BT, tissue samples were collected. A midline laparotomy was performed and MLN, spleen, and liver specimens were obtained under sterile conditions. Then, samples of ileum were removed for biochemical evaluation.

2.4. Microbiological analysis

Microbiological analysis was performed as described previously by Isenberg [18]. A sample of blood (1 mL) from each animal was immediately placed into Bactec Peds Plus/F blood culture medium (Becton Dickinson Microbiological Systems, Cockeysville, MD, USA) and incubated at 37 °C for 7 days under aerobic conditions in a Bactec 9240 system. Broths were incubated at 35 °C until turbid, and the turbidity was adjusted to match that of a 0.5 Mc Farland standard (10^8 CFU/mL). By using normal saline, a 1/100 dilution of the suspension was made to give an adjusted concentration of 10^6 CFU/mL. From all cultures, subsequent subcultures were performed on blood agar, eosin–methylen blue (EMB) agar, and chocolate agar [18]. All samples were stained with acridine orange and Gram technique. The liver, spleen, and MLN specimens were placed into 2 mL of brain heart infusion (BHI) broth, weighed, and homogenized. These samples were the placed on blood agar and EMB agar. All cultures were incubated under aerobic and anaerobic condition and were examined at 24 and 48 h for presence of growth. The identification of bacterial species was performed by standart microbiologic methods. Colonization was expressed as the number of CFU per milliliter of homogenate (CFU/g).

2.5. Detection of MDA levels

One part of the tissue was homogenized in 10 parts of 15 mmol/l KCL for malondialdehyde (MDA) assay MDA, which is the end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Measurement of MDA content by TBA reactivity is the most widely used methods for assessing lipid peroxidation [19]. The principle of the method is based on measurement of the absorbance of the pink color produced by interaction of TBA with MDA at 530 nm. Values were expressed as nmol/g wet tissue weight.

Table 1
Incidence of bacterial translocation and quantitative results in tissue specimens

Groups ($n = 8$)	MLN		Spleen		Liver	
	Incidence	CFU/g	Incidence	CFU/g	Incidence	CFU/g
Sham	0/8 (0%)	≤0.17	0/8 (0%)	≤0.17	0/8 (%0)	≤0.17
Burn[a]	7/8 (87.5%)	5.24 ± 0.78	5/8 (62.5%)	3.89 ± 1.1	5/8 (62.5%)	3.45 ± 0.97
Pre-burn	2/8 (25%)	1.54 ± 0.89	2/8 (25%)	1.44 ± 0.83	1/8 (12.5%)	0.86 ± 0.69
Post-burn	2/8 (25%)	1.54 ± 0.89	1/8 (12.5%)	0.83 ± 0.66	0/8 (0%)	≤0.17

Values expressed as logarithm of geometric mean (CFU/g tissue) ± S.E.M.
[a] $P < 0.05$ compared other groups.

2.6. Measurement of Ileal tissue myeloperoxidase (MPO) activity

One part of the tissue was homogenized in 10 parts of hexadecyltrimethylammonium bromide (HETAAB) buffer for MPO activity assay. The determination of tissue MPO activity depends on the fact that it reduces dimethoxibenzidine. Ileal segments were homogenized in 0.5% HETAAB in10 nM 3-(*N*-morpholino) propane sulfonic acid and centrifuged at 15,000 × *g* for 40 min. An aliquot of supernatant was mixed with a solution of 1% (w/v) dimethoxybenzidine and 1 nM hydrogen peroxide. After a 30-min incubation, the reaction was stopped by adding 3 M HCI. Activity was measured spectrophotometrically as the change in absorbance at 410 nm at 37 °C by using a spectrophtometer [20]. Results are expressed as units MPO activity per gram tissue.

2.7. Measurement of tissue glutathione levels

Each specimen (wet weight, 60 mg) was transferred to a glass homogenizer, and homogenized in 5% NaCl and extracted with 0.3 mL of ice-cold 60 g/l metaphosphoric acid and 1 mmol/l EDTA, and rapidly homogenized while the protein was directly precipitated. After centrifugation (3000 × *g* for 5 min at 4 °C), aliquots of the clear supernatants were either derivatized and analyzed promptly or stored for stability evaluation at −60 °C until analysis. The precipitate was analyzed for the concentrations of protein. 0.2 ml of the supernatant was mixed with 0.22 mmol/l NaH_2PO_4 and 0.1 mmol/l DTNB (5,5′-dithio-bis(2-nitrobenzoic acid). The mixture was vortexed and incubated for 5 min at room temperature. DTNB is a disulfide compound readily reduced by sulphidryl compounds that form a highly colored yellow anion. The optical density of this yellow substance is measured at 412 nm [21].

2.8. Statistical analysis

The statistical evaluation for proportional comparisons for quantitative cultures and biochemical values was done by using one-way ANOVA followed by Tukey test. Logarithmic values of quantitative cultures values were used in the analysis. Data were presented as mean ± S.E.M. for microbiologic results and mean ± S.D. for biochemical analysis. *P*-values less than 0.05 were considered as statistically significant.

3. Results

All animals survived until the completion of the experimental protocol. The incidence of BT within the groups is summarized in Table 1. BT did not occur in the sham group. Burn caused severe BT in group II, and the incidence of bacteria isolated from MLN, spleen, and liver was significantly higher in that group than the other groups ($P < 0.05$). NAC supplementation prevented BT in both pre-burn and post-burn groups. The predominating bacteria was *Escherichia coli*, however, *Klebsiella* and *Proteus vulgaris* were also encountered (data not shown).

The results of the biochemical analysis in tissue specimens are presented in Table 2. MDA levels were significantly increased in the burn group in comparison to sham, pre-burn, and post-burn groups ($P < 0.05$). MDA levels were significantly reduced in the both pre- and post-burn groups with NAC supplementation ($P < 0.05$) (Fig. 1). Thermal injury caused a significant increase in the levels of tissue MPO in burn group when compared with the other groups ($P < 0.05$).

Table 2
The results of biochemical analysis of tissue samples

Parameters	Sham ($n = 8$)	Burn ($n = 8$)	Pre-Burn ($n = 8$)	Post-Burn ($n = 8$)
MDA (nmol/ml)	38.22 ± 2.52	92.28 ± 61.6[a]	47.82 ± 22.89	48.79 ± 21.14
GSH	0.1982 ± 0.0583	0.0977 ± 0.041[b]	0.1210 ± 0.0321	0.1539 ± 0.0397
MPO (U/g)	0.0990 ± 0.03	0.02546 ± 0.0591[c]	0.0984 ± 0.0529	0.1443 ± 0.0429

Values are expressed as mean ± S.D.
[a] $P < 0.05$, significantly different from other groups.
[b] $P < 0.05$, significantly different from sham and post-burn group.
[c] $P < 0.05$, significantly different from other groups.

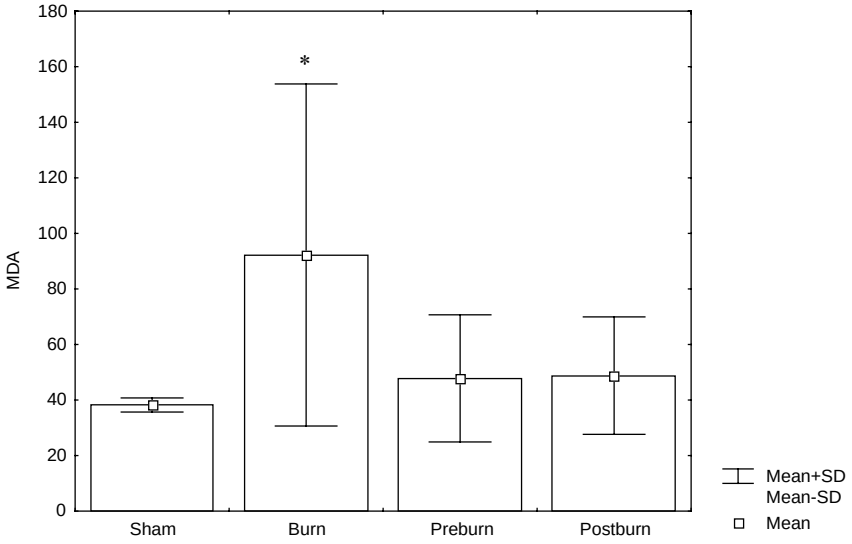

Fig. 1. Tissue MDA levels. Data expressed as mean ± S.D. (*) $P < 0.05$ significantly different from the other groups.

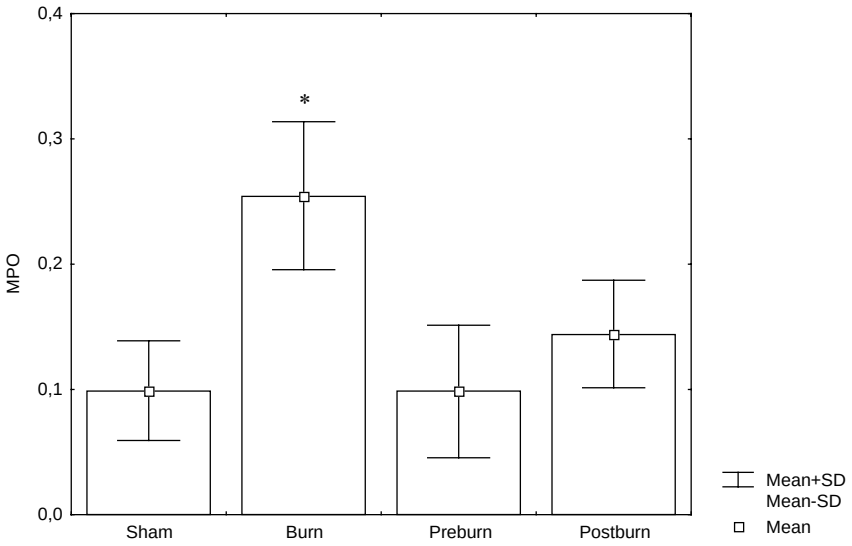

Fig. 2. Tissue MPO levels. Data expressed as mean ± S.D. (*) $P < 0.05$ compared to other groups.

Increased level of MPO in burn group was suppressed by NAC administration in the pre-burn and post-burn groups ($P < 0.05$) (Fig. 2). Also, burn injury caused significant decreases in tissue GSH levels when compared to the sham group ($P < 0.05$). GSH levels were preserved by post-burn NAC treatment and they were not significantly different from the sham group. Although, GSH levels in the pre-burn group were higher than that in the burn group, the difference was not statistically significant (Fig. 3, Table 2).

4. Discussion

The present study demonstrates that NAC prevents burn-induced BT while significantly decreasing the elevated lipid peroxidation and MPO activity in intestinal tissue after thermal injury, and it also maintains GSH levels. NAC prevented BT when applied 15 min before or 2 h after thermal injury. These results confirm the role of oxidative stress in intestinal mucosal barrier dysfunction and BT following burn injury.

Several studies have shown that the mesenteric blood flow decreases by more than 50% in the first 8 h after a severe thermal injury and rises back to the control levels after nearly 20 h [5,6]. The main cause of the decreased mesenteric blood flow is a transient and selective vasospasm in splanchnic area, and this post-burn mesenteric vasospasm results in I/R injury to intestinal mucosa leading to increased BT. In addition, the maximal incidence of BT occurs in the first 24 h after burn [22,23]. Our microbiological results which obtained

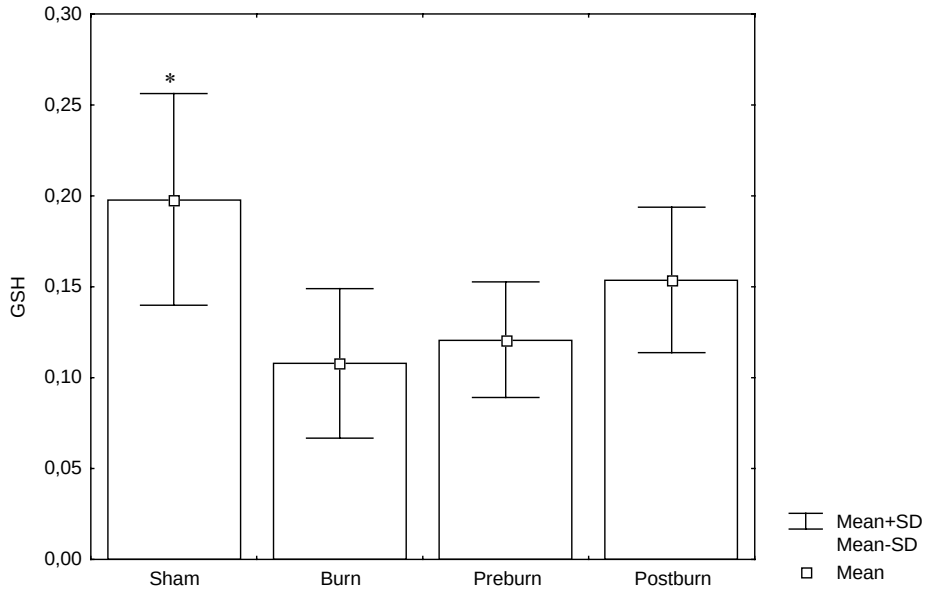

Fig. 3. Tissue GSH levels. Data expressed as mean ± S.D. (*) $P < 0.05$ compared to burn and pre-burn groups.

at 24 h after insult confirmed these reports. In concordance with the findings of BT in our previous intestinal I/R studies [24,25], the role of NAC in preventing burn-induced BT is most likely related to oxidant–antioxidant balance in I/R injury.

Ischemia and consecutive reperfusion cause oxidative stress, which is characterized by an imbalance between reactive oxygen species and the antioxidative defence system [26]. Potential mechanisms causing the development and progression of I/R injury include the adhesion and activation of polymorphonuclear neutrophils, the release of proinflammatory cytokines, as well as the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [27]. In many studies it has been showed that burn injury is associated with lipid peroxidation, which is believed to be an important cause of oxidative damage to cellular membranes, and eventually cell death [28]. Recently, Horton revealed clearly the role of ROS and RNS on lipid peroxidation in burn injury [29]. MDA is a good indicator of oxidative injury and an end product of lipid peroxidation. Several studies have demonstrated that burn injury and I/R injury are associated with elevated levels of MDA in different organ and tissues [15,28,30]. In our study, the levels of MDA significantly increased in intestinal tissue at 24 h after the thermal injury. These elevated levels were reversed back to control levels by NAC supplementation in both pre- and post-burn group. This protective effect of NAC largely depends on the findings of increased levels of GSH emphasizing the role of antioxidants in our study groups. Additionally, it may be due to by either directly interfering with ROS, such as OH^-, or enhancing antioxidant systems, such as superoxide dismutase or glutathione peroxidase [31].

Non-protein sulfhydryl containing compounds, especially GSH, one major constituent of cellular defence mechanisms against oxidative stress. GSH acts either directly or via glutathione peroxidase catalysis to scavenge the generated ROS [32]. Tissue ischemia depletes intracellular GSH levels, and this is probably due to excess GSH consumption during oxidative stress. Several reports indicate that GSH levels of various tissues including lung, liver, kidney, and intestine were significantly decreased after thermal and I/R injury [14,15,28]. NAC is a thiol compound and contains a sulfhydryl group, therefore, it is known as a GSH precursor. It has been shown that NAC exerts its antioxidant effect in two ways. First of all, as a source of sulfydryl groups it indirectly facilitates GSH biosynthesis and, hence, increase GSH supply for glutathione peroxidase [9]. Secondly, it directly reacts with ROS [9]. Overall decrease in the oxidative damage by above-mentioned mechanisms might also have decreased the consumption of GSH. There are several studies which have demonstrated that NAC therapy improves GSH levels in lung at 24 h after burn [33] and in liver and kidney in I/R injury [14,15]. In accordance with these data, our findings also verify that NAC maintained GSH levels while it prevented against burn-induced oxidative stress.

Reduction of lipid peroxidation also paralleled with the change in MPO activity, which is known as the index of infiltration of polymorphonuclear neutrophils (PMN). PMN are a potential source of ROS, and have a major role in development of oxidative tissue injury. Experimental studies have been shown that MPO activity increases in several inflammatory processes, such as I/R injury [10], burn injury [28], and CLP-induced sepsis [16]. In a recent study, it has been demonstrated that MPO activity increases in lung, liver, and intestine at 24 h after burn injury [28]. Cuzzocrea et al. showed that MPO activity decreased with NAC treatment in the ileal tissue [10]. In parallel all these, we found MPO activity elevated in burn group at 24 h after burn in the present

study. Furthermore, also NAC supplementation reduced the MPO activity in the both treatment groups.

On the other hand, several mechanisms have been postulated for this anti-inflammatory effect of NAC. ROS and/or RNS, generated by neutrophils, activates NF-κB, a transcriptional regulatory protein, resulting in the excessive production of inflammatory cytokines, including interleukin-8 (IL-8) and tumor necrosis factor (TNF), in multiple organ dysfunction associated with I/R injury [34]. It has been shown that NAC treatment suppresses NF-κB activation in a model including endotoxin-mediated oxidative stress [35]. Similarly, NAC supplementation might have suppressed NF-κB activation in our study. Another possible mechanism is the alteration of interactions between leukocytes and endothelial cells which are mediated by different adhesion molecules. P-selectin is a member of selectin family, and mediates rolling of leukocytes along the endothelium. Intercellular adhesion molecule-1 (ICAM-1), which is one of the most important ligands for leukocytes, mediates binding of leukocytes on the endothelium during the reperfusion. P-selectin is rapidly released to the cell surface after exposure to certain stimuli, such as ROS and expression of ICAM-1 can be enhanced by various cytokines, such as IL-1 and TNF. Consequently, these molecules are considered to play a crucial role of in inflammatory processes [36,37]. Cuzzocrea et al. have previously demonstrated that NAC treatment abolished the expression of P-selectin and upregulation of ICAM-1, together with the elevation in MPO activity, in a splanchnic artery occlusion shock model [10]. These findings suggest that NAC treatment can interrupt with the interaction of leukocytes and endothelial cells both during the rolling phase mediated by P-selectin and adhesion phase mediated by ICAM-1. In the present study, although we did not study these adhesion molecules, NAC might also have reduced the expression of P-selectin and upregulation of ICAM-1 in both NAC supplementation groups. On the other hand, despite increasing amount of experimental data regarding the anti-inflammatory effects of NAC in several inflammatory processes, such as I/R injury and CLP-induced sepsis, Molnar suggested that NAC treatment had no potential benefit in critical illness [38]. Thus, the issue still remains controversial.

In conclusion, thermal injury, which is a cause of oxidative damage in small intestine, induces intestinal mucosal dysintegrity and BT. NAC treatment maintained the intestinal mucosal barrier integrity and prevented BT after burn injury. We believe that use of NAC merits consideration as a therapeutic agent in burn injury due to its antioxidant and GSH supplier effects.

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