Tissue oxidative stress level and remote organ injury in two-hit trauma model of sequential burn injury and peritoneal sepsis are attenuated with N-acetylcysteine treatment in rats

Ardışık yanık ve peritoneal sepsisle oluşturulan iki darbeli travma modelinde, N-asetilsistein tedavisi doku oksidatif stres düzeyini ve uzak organ hasarını azaltır

Ahmet GÜRER,[1] Mehmet ÖZDOĞAN,[1] Ali Kağan GÖKAKIN,[1] İsmail GÖMCELİ,[1] Özlem GÜLBAHAR,[2] Ata Türker ARIKÖK,[3] Hakan KULAÇOĞLU,[3] Raci AYDIN[1]

BACKGROUND

The second hit in trauma leads to an exaggerated inflammatory response and multiple organ failure. Infection following burn injury is a useful model for two-hit trauma studies. The aim of this study was to investigate the effect of N-acetylcysteine (NAC) treatment as an antioxidant in a two-hit trauma model.

METHODS

30% scalding burn injury was performed in 45 rats and cecal ligation-puncture (CLP) was performed 72 hours later. Groups were allocated as follows: Group I: No treatment was performed; Group II: Rats were treated with 150 mg/kg/day i.p. NAC for 72 h following CLP; Group III: Rats were treated with 150 mg/kg/day i.p. NAC for 6 days following thermal injury. Tissue samples were collected to study the tissue malonyldialdehyde (MDA) and glutathione (GSH) levels, and for histopathological examination on day 7.

RESULTS

No difference in mortality between groups was detected. Tissue MDA levels significantly decreased in the liver (p=0.01, p=0.02) and ileum (p=0.01, p=0.02) in the treatment groups. Lung tissue GSH levels were found to be significantly increased in Group II (p=0.02). Lung injury scores were decreased in Group II (p=0.005) compared to the control group.

CONCLUSION

NAC attenuated tissue oxidative stress level and remote organ injury in two-hit trauma. Further experimental and clinical studies on this subject are necessary.

Key Words: Antioxidant; burn; cecal ligation and puncture; N-acetylcysteine; trauma; two-hit; peritonitis.

AMAÇ

Travma hastalarında ikinci darbenin oluşması enflamatuvar yanıtın şiddetlenmesine ve çoklu organ gelişmesine neden olmaktadır. Yanık hasarı sonrası oluşan enfeksiyon iki darbeli travma çalışmaları için uygun bir modeldir. Bu çalışmada, ardışık yanık ve sepsisten oluşan iki darbeli travma modelinde N-asetilsistein'in (NAS) etkinliği araştırıldı.

GEREÇ VE YÖNTEM

Kırk beş sıçanda %30'luk oranda haşlanma yanığı oluşturulduktan 72 saat sonra çekal bağlama ve delme yöntemi ile peritonit oluşturuldu. Grup I'de herhangi bir tedavi uygulanmadı. Grup II'deki sıçanlara peritonit sonrasında 150 mg/kg/gün dozunda intraperitoneal NAS tedavisi verildi. Grup III'e yanık sonrası 6 gün boyunca 150 mg/kg/gün dozunda NAS tedavisi uygulandı. Yedinci gün doku malondialdehit (MDA) ve glutatyon (GHS) düzeylerinin tayini ve histopatolojik hasarın değerlendirilmesi için örnekler toplandı.

BULGULAR

Gruplar arasında mortalite farkı yoktu. Tedavi gruplarında karaciğer ve ileum doku MDA düzeylerinde anlamlı azalma (p=0,01, p=0,02) saptandı. Grup II'de akciğer GSH düzeylerinin anlamlı olarak arttığı (p=0,02) ve akciğer dokusunda histopatolojik hasar skorunun azaldığı saptandı (p=0,005).

SONUÇ

N-asetilsistein tedavisi çalışılan iki darbeli travma modelinde doku oksidatif stres düzeyini ve uzak organ hasarını hafifletmiştir. Bu konuda daha ileri deneysel ve klinik çalışmalara ihtiyaç vardır.

Anahtar Sözcükler: Antioksidan; yanık; çekal bağlama ve delme; N-asetilsistein; travma; iki darbe; peritonit.

[1]Department of General Surgery, Atatürk Training and Research Hospital; [2]Department of Biochemistry, Gazi University, Faculty of Medicine; [3]Department of Pathology, Dışkapı Yıldırım Beyazıt Training and Research Hospital, Ankara, Turkey.

[1]Atatürk Eğitim ve Araştırma Hastanesi, Genel Cerrahi Kliniği; [2]Gazi Üniversitesi Tıp Fakültesi, Biyokimya Anabilim Dalı; [3]Dışkapı Yıldırım Beyazıt Eğitim ve Araştırma Hastanesi, Patoloji Kliniği, Ankara.

Correspondence *(İletişim):* Mehmet Özdoğan, M.D. Bilkent yolu, No: 2, 06500 Ankara, Turkey.
Tel: +090 - 312 - 291 25 25 Fax *(Faks):*+090 - 312 - 291 27 05 e-mail*(e-posta):* mehmetozdogan01@superonline.com

Sepsis remains a major cause of morbidity and mortality in intensive care units (ICUs). Reactive oxygen species (ROS) play an important role in inflammatory processes as mediators of injury.[1] During hypoxic and acidotic conditions, and in the presence of endotoxin, not only the reduction of oxygen but also the activation of white blood cells leads to the production of ROS.[2] ROS are believed to be important mediators of cellular injury contributing to the development of sepsis.[3] The hydroxyl radical scavengers were found to dose-dependently inhibit pro-inflammatory cytokine production.[4]

Lipid peroxidation caused by ROS has a great role in thermal injury. Ischemia-reperfusion injury due to transient splanchnic vasoconstriction following major burns causes oxidative damage in intestinal tissue. Thus, burn injury leads to breakdown in the intestinal mucosal barrier, which can induce bacterial translocation.[5,6]

One of the experimental therapeutic approaches for the inhibition of oxidant-mediated injury is the use of glutathione (GSH)-modulating agents like thiol or sulfhydryl compounds. Among them, N-acetylcysteine (NAC) is one of the most widely investigated agents that serves as a precursor of GSH and also acts as a direct scavenging agent. In experimental models, it was shown that NAC treatment inhibits the production of cytokines and adhesion molecules, reduces bacterial translocation and pulmonary ROS production, and improves survival.[6-9]

The two-hit trauma model states that the second hit, mostly superimposing infection, has an additive effect on the consequences of the original first trauma, leading to an exaggerated systemic inflammatory response and multiple organ failure.[10,11] Infection following burn injury is a common clinical condition and a useful model for two-hit trauma studies. The aim of this study was to investigate the effect of NAC treatment on oxidative stress and organ injury in a burn and sepsis two-hit trauma model.

MATERIALS AND METHODS

A total of 45 adult male Wistar rats weighing between 180 to 250 g were included in the study. The animals were fed with standard diet and water ad libitum during the experiment. All animals were housed separately and kept under standard conditions of room temperature (22-24 °C) and a 12-h light/12-h dark cycle. Animals were fasted for 12 h before the experiment. The Institutional Committee of Ethics of Animal Experiments at Ankara Education and Research Hospital approved the study in accordance with the Declaration of Helsinki and the Institution's guide for the use of laboratory animals.

Experimental design

Animals were divided into three groups of 15 rats each. A standard 30% scalding burn injury was performed to rats in each group and 72 h after thermal injury, secondary peritonitis was induced by cecal ligation and puncture (CLP) procedure.

Groups were designated as follows: Group I: No treatment was performed; Group II: Rats were treated with 150 mg/kg/day intraperitoneal (i.p.) NAC for 72 h following CLP procedure; Group III: Rats were treated with 150 mg/kg/day i.p. NAC for 6 days following thermal injury. Animals were sacrificed on the seventh day of the experiment. Samples of lung, liver and ileum tissue were collected to study the levels of tissue malonyldialdehyde (MDA) and GSH, and for histopathological examination.

Anesthesia

Animals were anesthetized with i.p. xylazine (5 mg/kg) and ketamine (30 mg/kg) during the scalding and CLP procedure.

Burn injury

A 3x4 cm area on the back sides of the rats was shaved to obtain 30% naked area. The rats were then placed in a bar that only permits exposure of the shaved area. To obtain a third-degree standard scalding burn injury, the shaved areas of mice were placed in 96 °C boiling water for 12 seconds.

Cecal ligation and puncture

After performing a midline laparotomy under general anesthesia, the cecum was isolated and ligated via 3/0 silk just distal to the terminal ileum. The cecum was then perforated at two different points by 22 gauge needle. The cecum was squeezed for fecal discharge and to obtain contamination. Four ml of normal saline was infused i.p. for fluid resuscitation and the abdomen was then closed in a routine manner.

Tissue MDA levels

The tissue MDA levels of the lung and liver were evaluated by the method described by Uchiyama-Mihara.[12] The color formed at the end of the reac-

Table 1. Tissue MDA and GSH levels

	MDA (nmol/g-tissue)			GSH (µmol/mg-tissue)	
	Liver	Lung	Ileum	Liver	Lung
Group I	80.7±6.4	20.6±4.1	19±2.6	1.3±0.4	0.05±0.004
Group II	46.3±5.8	20.3±7.3	7.8±2.0	2.0±0.9	0.09±0.005
Group III	32.4±6.2	20.2±1.9	5.8±0.8	2.3±0.2	0.05±0.004

tion of thio-butyric acid (TBA) and MDA was evaluated using this method, and 1.15% KCl, 1% phosphoric acid, 0.6% TBA and n-butanol reactants were used. 200 mg tissue homogenized in 1.8 ml KCl and 3 ml phosphoric acid and 1 ml TBA were added to 0.5 ml homogenized solvent. This mixture was left in boiling water for 45 min and cooled just after this period. Butanol (4 ml) was then added and centrifuged at 3500/min for 10 min. The butanol phase at the upper side was separated and results were defined on spectrophotometer in 535 and 520 nm wavelengths. The results were defined as nmol/g-tissue after calculations.

Tissue GSH levels

The tissue GSH levels of the lung and liver were evaluated by the method as described by Elman,[13] including metaphosphoric acid (0.5 M), $KHPO_4$ buffer 1 M (pH 8), and DNPH (dinitrophenylhydrazine) as reactant. A piece of tissue was homogenized with 1 ml metaphosphoric acid and centrifuged at 3500/min for 10 min. GSH and protein levels were evaluated at the supernatant side. The color formed by the reaction of GSH and DNPH in buffer at the supernatant side was evaluated on spectrophotometer in 410 wavelength and the results after calculations were expressed as µmol GSH/mg-protein.

Histologic and pathologic evaluation

Five micrometer-thick sections were cut from specimens, fixed in formalin, embedded in paraffin, and then painted by hematoxylin-eosin (H-E). Preparations were evaluated under light microscope (X400). To define the lung injury, the neutrophil infiltration and constitutional injury ratio were evaluated. Normal lung tissue was defined as score 1, presence of neutrophil infiltration as score 2, neutrophil infiltration with a constitutional injury less than 50% as score 3, and neutrophil infiltration with a constitutional injury more than 50% as score 4. Regarding the injury in liver tissues, sinusoidal congestion, vegetative degeneration and the ratio of necrosis were taken into account. Up to 25%, 25%-50%, and more than 50% injury for each parameter was rated as score 1, score 2, and score 3, respectively.

Statistical analysis

All numerical data were expressed as mean ± SEM. Statistical analysis was made by using SPSS 11.5 program. Kruskal-Wallis test was performed for assessing the statistical differences between independent groups, and Mann-Whitney U-test was performed for assessing inter-group differences. A value of $p<0.05$ was considered statistically significant.

RESULTS

During the experimental period, 5, 5 and 4 mortalities were observed in Group I, Group II, and Group III, respectively. All mortalities were seen in the first 72 hours. There was no statistically significance difference in mortality between groups.

Tissue MDA levels

The tissue MDA levels detected in the lung, liver and ileum and comparisons between the groups are shown in Table 1 and Fig. 1. A statistically significant decrease was found in tissue MDA levels of the

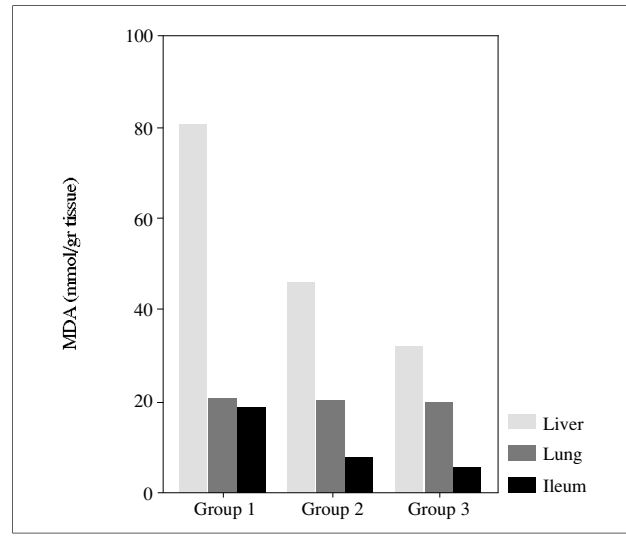

Fig. 1. Tissue MDA levels in groups.

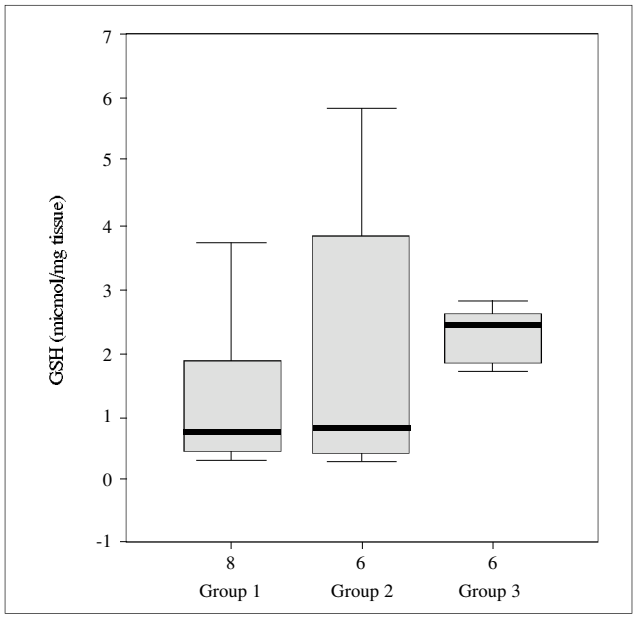

Fig. 2. GSH levels in liver tissue.

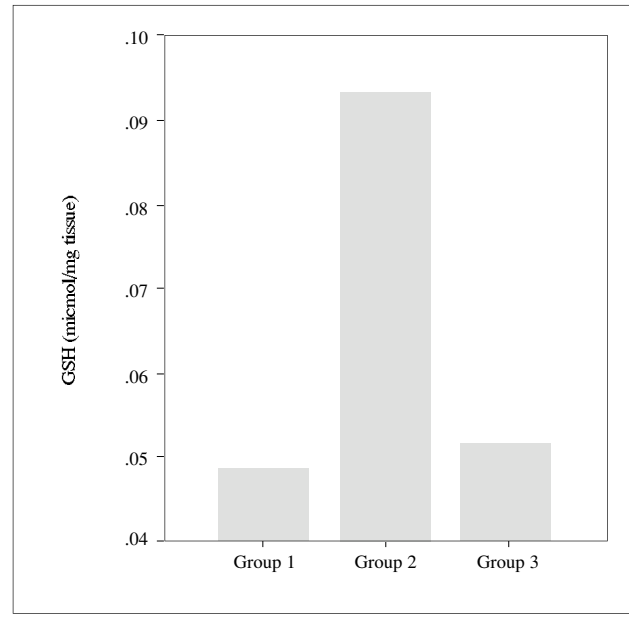

Fig. 3. GSH levels in lung tissue.

liver (p=0.01) and ileum (p=0.01) in Group II compared to Group I. Similarly, there was a statistically significant decrease in tissue MDA levels of the liver (p=0.02) and ileum (p=0.02) in Group III compared to the control group. There were no statistically significant differences in MDA levels detected in lung tissues of Group II and Group III when compared to Group I. No statistical difference was found in tissue MDA levels between Group II and Group III.

Tissue GSH levels

The GSH levels in ileum tissues could not be evaluated for technical reasons. The tissue GSH levels detected in the lung and liver are shown in Table 1 and the comparisons between the groups are shown in Fig. 2 and Fig. 3. Lung tissue GSH levels were found to be significantly increased in Group II compared to Group I (p=0.02); however, there was no significant difference regarding liver GSH levels. A statistically significant increase was detected in tissue GSH levels of the liver (p=0.002) in Group III compared to Group I, but no difference was found in GSH levels in lung tissue.

Histopathological findings

There was no statistically significant difference between the groups when the liver injury scores were compared. It was found that lung injury scores were statistically decreased in Group II (p=0.005) compared to Group I (Fig. 4). No statistically significant difference was detected in Group III. A trend towards increased lung injury score was detected in Group III compared to Group II, but this trend did not reach statistical significance (p=0.054).

DISCUSSION

It is known that the two-hit trauma model is effective in the occurrence of systemic complications like adult respiratory distress syndrome following trauma.[14-17] The common components of sepsis, such as increase in lactate levels, thrombocytopenia, and hypodynamic-hyperdynamic shock are detected more often in the two-hit trauma models.[18]

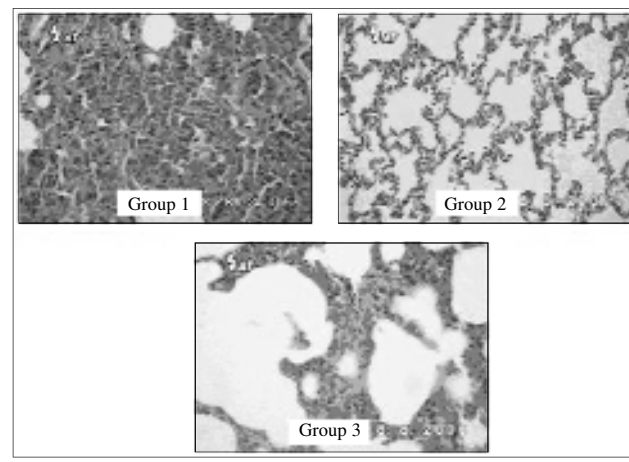

Fig. 4. Histopathological findings in liver tissue. Dense neutrophil infiltration and alveolar destruction were observed in Group I. These findings were attenuated in Group II.

It was previously shown in a two-hit trauma model of burn injury followed by CLP that first hit (burn injury) attenuated resistance against peritoneal sepsis and increased mortality.[19] In this model, the second hit (CLP) caused maximum mortality after the 7th day when compared to the burn injury-only group. These findings are consistent with the susceptibility of burn injury patients to infections after the first week of the injury.[19-22]

Burn injury results in significant third space fluid loss with accompanying splanchnic vasoconstriction that leads to gut mucosal ischemia. Fluid resuscitation after burn injury causes mucosal ischemia/reperfusion injury resulting in excessive ROS production.[5,23-25] It has been proposed that oxidative stress during inflammatory conditions results in over-consumption of GSH in liver and lung tissue. Antioxidants, such as NAC and GSH, reduced oxidative stress and diminished tissue injury in experimental burn injury studies.[26] Administration of NAC as a precursor of GSH in burn injury has been shown to improve cellular immunity, attenuate mucosal damage and reduce increased lung and intestinal MDA levels.[6,8,27]

Clinical studies in patients with septic shock demonstrated that NAC treatment diminished lipid peroxidation, stabilized hemodynamic findings by improving cardiac contractility, and shortened mechanical ventilation and total ICU period.[28,29] However, these studies did not find a significant effect on mortality.

The present study aimed to investigate the effectiveness of NAC treatment on the two-hit trauma model, which can resemble clinical sepsis. To our knowledge, no previous experimental study in the English literature has investigated the effect of antioxidant therapy on the two-hit trauma model.

We found that NAC treatment attenuated oxidative stress in the liver and ileum tissue in this sequential burn injury and CLP peritonitis two-hit trauma model. Short-term NAC treatment showed a protective effect in lung tissues, increasing GSH levels. Koksel et al.[30] found that long-term (7 days) NAC administration decreased lipid peroxidation and histopathological injury in lung tissue in a model of CLP sepsis. In contrast to their findings, long-term NAC did not prevent lung damage and did not increase lung tissue GSH levels in the present study. Aggravated pulmonary neutrophil infiltration and tissue injury after the second hit were attenuated by short-term NAC treatment. Similarly, NAC treatment improved GSH levels in the liver tissue.

In conclusion, NAC treatment attenuated tissue oxidative stress level and pulmonary remote organ injury in significant two-hit trauma. NAC treatment is a promising adjunctive modality in clinical two-hit trauma and multiple organ failure sequence models, such as burn, hemorrhagic shock and sepsis. Major trauma patients may benefit from NAC treatment that is commenced before the second hit, mostly with respect to infective complications due to immunosuppression. Further experimental and clinical studies are necessary on this subject.

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