Turkish Journal of Clinics and Laboratory

To cite this article: Tumer NB. N-acetyl cysteine attenuates ferroptosis mediated lung injury induced by lower limb ischaemia/reperfusion.Turk J Clin Lab 2020; 4: 288-293.

Original Article

N-acetyl cysteine attenuates ferroptosis mediated lung injury induced by lower limb ischaemia/reperfusion

N-asetil sistein alt ekstremite iskemi/ reperfüzyonu tarafından indüklenen feroptosise bağlı akciğer hasarını azaltır

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Abstract

Aim: This study aimed to analyze the effect of N-acetyl cysteine pretreatment on the prevention of ferroptosis mediated lung injury induced by lower limb ischemia and reperfusion.

Material and Methods: Eighteen male Sprague-Dawley rats weighing 350-400 g were randomized into three groups. The animals received N-acetyl cysteine 150 mg/kg or normal saline 0.1 ml/kg intraperitoneally before the ischemic period. In the control and study groups, I/R injury was induced by clamping the aorta infrarenal for 2 hours, followed by 4 hours of reperfusion. The third group underwent sham surgery. After sacrifice, the lungs of the animals were extracted for both histopathological and biochemical analysis.

Results: There was a significant difference between the control and study animals regarding tissue malondialdehyde (MDA), and glutathione (GSH) levels. In the control group, the MDA levels were increased and the GSH levels were increased significantly compared to the sham group that revealed a ferroptosis mediated lung injury. However, N-acetyl cysteine decreased the levels of MDA and increased the levels of GSH revealing a protective effect. The Prussian blue (free iron stain) staining which was used to examine iron deposition revealed a reduced deposition of iron in the N-acetyl cysteine group.

Conclusion: The results of the present study suggest a protective effect of N-acetyl cysteine on ferroptosis mediated lung injury induced by lower limb ischemia-reperfusion in a rat model.

Keywords: Feroptosis; ischemia/reperfusion; n-acetyl cysteine

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Recevied: 15.07.2020 accepted: 11.08.2020
Doi: 10.18663/tjcl.769961

Öz

Amaç: Bu çalışma, N-asetil sistein ön tedavisinin, alt ekstremite iskemisi ve reperfüzyonun neden olduğu ferroptoza bağlı akciğer hasarının önlenmesi üzerindeki etkisini analiz etmeyi amaçlamıştır.

Gereç ve Yöntemler: 350-400 g ağırlığında on sekiz erkek Sprague-Dawley sıçanı üç gruba randomize edildi. İskemik dönemden önce intraperitonal olarak 150 mg / kg N-Asetil Sistein veya normal salin 0.1 ml / kg verildi. Kontrol ve çalışma gruplarında, aort infrarenal düzeyde klemlenerek 2 saat boyunca I/R ve sonrasında 4 saat reperfüzyon indüklendi. Üçüncü gruba sham grubu olarak kullanıldı. Hayvanlarının hayatı sonlandırıldıktan sonra, histopatolojik ve biyokimyasal analiz için akciğerleri çıkarıldı.

Bulgular: Doku Malondialdehit (MDA) ve glutatyon (GSH) seviyeleri arasında istatistiksel olarak anlamlı fark tespit edildi. Kontrol grubunda, MDA düzeyleri ve GSH düzeyleri, ferroptoza bağlı akciğer hasarı gösteren sham grubuna kıyasla önemli ölçüde arttı. Bununla birlikte, NAC alan grupta MDA seviyesi düşerken koruyucu bir etki olarak GSH seviyeleri yükseldi. Demir birikimini göstermek için kullanılan Prusya mavisi (serbest demir lekesi) boyaması, N-asetil sistein grubunda demir birikiminin azaldığını ortaya çıkarmıştır.

Sonuç: Bu çalışmanın sonuçunda, bir sıçan modelinde alt ekstremite iskemisi-reperfüzyonunun neden olduğu ferroptozise bağlı akciğer hasarı üzerine N-asetil sisteinin koruyucu bir etkisi olduğunu düşündürmektedir.

Anahtar kelimeler: Feroptozis; iskemi/reperfüzyon; n-asetil sistein

Introduction

Lower limb ischemia/reperfusion (I/R) is an important and common event in clinical practice. Reperfusion results in both local and systemic damage, in part through rapid oxygen free radical generation and inflammatory mediators [1,2]. Reactive oxygen species (ROS) have a destructive role in mediating tissue damage during I/R injury. Once the reestablishment of blood flow to the ischaemic tissue is provided, an influx of molecular oxygen catalyzes xanthine oxidase to degrade hypoxanthine to uric acid and thereby liberating the highly reactive superoxide anion (O2-). Superoxide is then converted to hydrogen peroxide (H2O2) and the hydroxyl radical (OH•). The major consequence of hydroxyl radical production is peroxidation of the lipid structures of cell membranes resulting in cell death [2]. Remote organ damage after I/R mainly occurs in the lungs, kidneys, and heart.

Ferroptosis is a form of regulated cell death identified as iron-dependent, nonapoptotic cell death. In addition to iron accumulation, there is also an accumulation of lipid peroxidation products in this type of cell death [3]. In ferroptosis, there is also depletion of glutathione (GSH) or the inactivation of the lipid repair enzyme GSH peroxidase 4 (GPx4) [4]. The major factor that triggers ferroptosis is ROS accumulation [5]. Ferroptosis has been reported to be involved in many pathophysiological situations such as myocardial infarction, degenerative diseases, neurological diseases, antiviral immunity, cancer, and I/R injury [6-9]. It is reported that inhibition of ferroptosis alleviated I/R induced acute lung injury [9,10].

N-acetylcysteine (NAC) is a sulfhydryl group transmitter. It is used primarily in the treatment of paracetamol overdose [11]. It is a strong antioxidant, mucolytic and has anti-inflammatory effects. NAC as an antioxidant directly scavenges ROS through its thiol groups and increases intracellular glutathione levels [12].

Therefore, this study aimed to analyze the effect of NAC pretreatment on the prevention of ferroptosis mediated lung injury induced by lower limb ischemia and reperfusion.

Materials and Methods

This study was approved by the Institution of Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. (IRB No: 66332047-604.01.02) Eighteen male Sprague-Dawley rats weighing 350-400 g were randomized into three groups. The animals were initially anesthetized with intraperitoneal ketamine hydrochloride (Ketalar; Pfizer, Ortakoy, Istanbul, Turkey) 100 mg/kg body weight. The abdomen was then explored through a midline incision after shaving and disinfection. In the sham group (Group I), laparotomy was performed. In the control group and the study groups, I/R injury was induced by clamping the aorta with atraumatic vascular clamp infrarenal for 2 hours, followed by 4 hours of reperfusion. Cessation of arterial flow

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was confirmed using the absence of an audible continuous-wave Doppler signal. Study group animals (Group II) received NAC 150 mg/kg and control group animals (Group III) received normal saline 0.1 ml/kg intraperitoneally before the ischemic period. At the end of these procedures, the animals were sacrificed with the lethal injection of sodium thiopental (Pentothal Sodium, Abbot, Italy). Immediately after sacrifice, through midline sternotomy, lungs were extracted and washed with 0.9% saline solution for both histopathological (hematoxylin-eosin staining) and Prussian blue staining (free iron stain, Sigma-Aldrich) to examine iron deposition and biochemical analysis (malondealdehyde assay (MDA) and total glutathione (GSH) assays).

Wet-to-dry lung weight ratio

Wet-to-dry lung weight ratio (W/D) is an index of pulmonary edema. The left lower lung was excised and weighed immediately (wet weight). The lung tissue was then dried in an oven for 5 days at 60°C and reweighed (dry weight). The W/D was calculated by dividing the wet weight by the dry weight, as described previously [13].

Biochemical Analysis

Lung tissues were frozen immediately in liquid nitrogen and stored at -80°C until measurements were done. We prepared 20-µm-thick sections, which were dried under vacuum overnight at 20°C. The dried-frozen sections were stored at -20°C until biochemical assays were done.

The determination of MDA and total GSH were performed by enzyme-linked immunosorbent (ELISA) assay. The levels of MDA in lung tissue were measured based on the Biotin double antibody sandwich technology (Bioassay Technology Laboratory, Shangai, China). MDA concentration was expressed as nmol/ml (level of detection (LOD): 0.024 nmol/ml, level of quantification (LOQ): 10 nmol/ml). Total GSH levels in the lung tissue were determined according to Ellman in nmol/mg [14].

Histopathological examination

Tissue samples were fixed in 10% formalin and embedded in paraffin with routine follow-up procedure; 4-5 µm sections were cut from paraffin blocks and stained with hematoxylin and eosin (H-E) and Prussian blue for light microscopic examination (x200). A histopathologist unaware of the groups assigned a score of 0–4 to each section as follows: 0, normal histologic appearance; 1, vascular congestion; 2, vascular congestion and interstitial edema; 3, alveolar structural disturbance and infiltration of inflammatory cells; 4, massive alveolar structural disturbance and infiltration of inflammatory cells [15]. Prussian blue staining is widely used for the assessment of iron deposition in tissues. After deparaffinization and rehydration, the lung sections were treated with a 20% hydrochloric acid solution to liberate ferric iron and then treated with a 10% aqueous potassium ferrocyanide solution to produce insoluble ferric ferrocyanide. After this procedure, the sections were counterstained with eosin. This staining identifies ferric iron as bright blue and nuclei as red [16].

Statistical analysis

Statistical analyses were performed using SPSS 18 (IBM Corp, New York, USA). Values are expressed as mean±SD. The difference between the groups was analyzed either with unpaired

Student's t-test or one-way ANOVA with Bonferroni's correction. p-Values of less than 0.05 were considered significant.

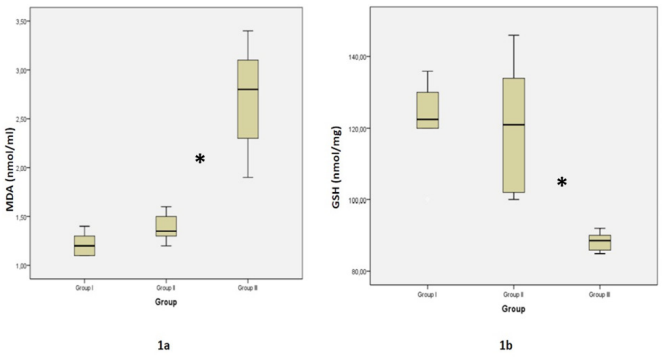


Figure 1. A: Malondialdehyde (MDA) production was reduced in NAC pretreated lung tissues (Group II) compared with that of the control group (Group III). B: Total GSH levels were increased in the NAC group. Group I: Sham group. Data are presented as medians with total range. *p<0.05 Group II vs Group III.

Results

Biochemical assay results

MDA was studied here as a marker of free radical-mediated lipid peroxidation. In the control group animals, the tissue MDA levels were increased significantly compared to the sham group that revealed a ferroptosis mediated lung injury (2.72±0.56 and 1.22±0.12 respectively, p< 0.001). Animals that received NAC showed a general trend of less lipid peroxidation product in lung tissue than the control group and this decrease was statistically significant (Figure 1a). Tissue MDA levels

significantly decreased in NAC received animals compared to the control group (1.38±0.15 and 2.72±0.56 respectively, p<0.001). N-acetyl cysteine pretreatment increased the tissue levels of GSH compared to the control group revealing a protective effect (120.67±19.17 and 88.33±2.58 respectively, p=0.002, Figure 1b).

Weight to dry ratio results

A higher W/D was noted after IR. The NAC pretreatment group had lower W/D (Figure 2).

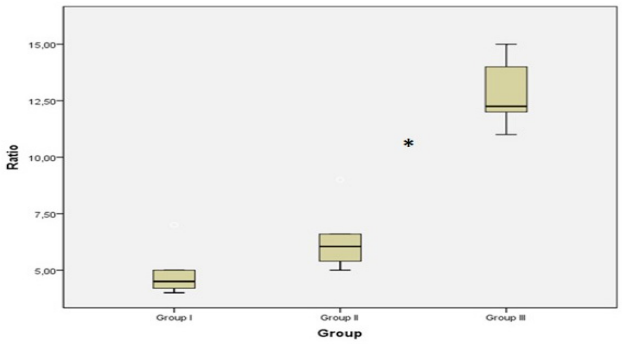


Figure 2. Lung wet-to-dry weight ratio. Edema formation (wet/dry weight) in lung tissue increased following lower extremity ischemia-reperfusion. Data are presented as medians with total range. *p < 0.05 Group II vs Group III. Group I: sham, Group II: NAC pretreatment, and Group III: control.

Histopathological examination results

Hemotoxylin and eosin staining examination showed normal lung architecture and arrangement in the sham group. The histological structure of pulmonary interstitial tissue and alveolus was intact and visible in the sham group by light microscopy, without infiltration of inflammatory cells stained with H-E (Figure 3a). Obvious histological changes were observed in the control group (Figure 3b) compared to the sham group: atelectasis, thickening of alveolar inter wall, infiltration of inflammatory cells. Histological changes decreased in the study group pretreated with NAC compared to the control group; NAC pretreatment protected against I/R induced injury, with approximately normal lung architecture in lung sections with the normal structure of pulmonary interstitial tissue and alveolus, and less infiltration of inflammatory cells (Figure 3c). In the control group, lung injury was significantly higher than the sham and NAC pretreatment groups (p<0.001; Figure 4). These results indicated that lung injury score was significantly increased by IR, whereas NAC pretreatment significantly decreased lung injury score (Figure 4). The Prussian blue staining which was used to examine iron deposition revealed no or very little amount of detectable iron in sham group lung sections (Figure 3d). However, intense blue positive staining was consistently observed in sections from control animals (Figure 3e), whereas the amount of stainable iron was significantly reduced in NAC pretreated animals (Figure 3f).

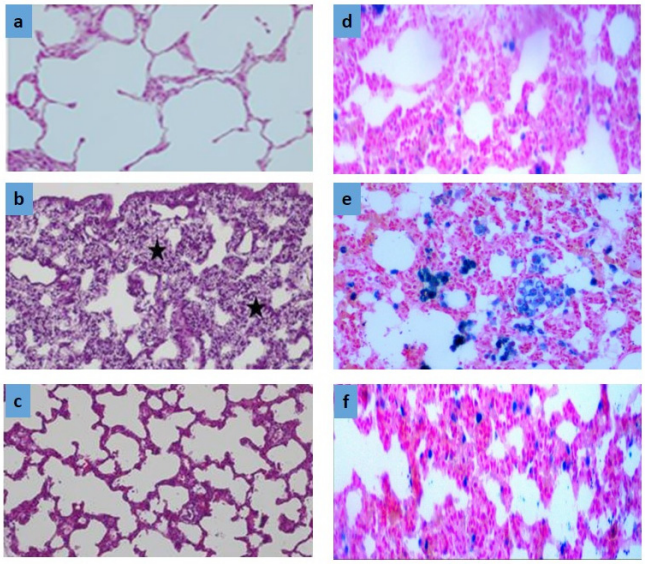
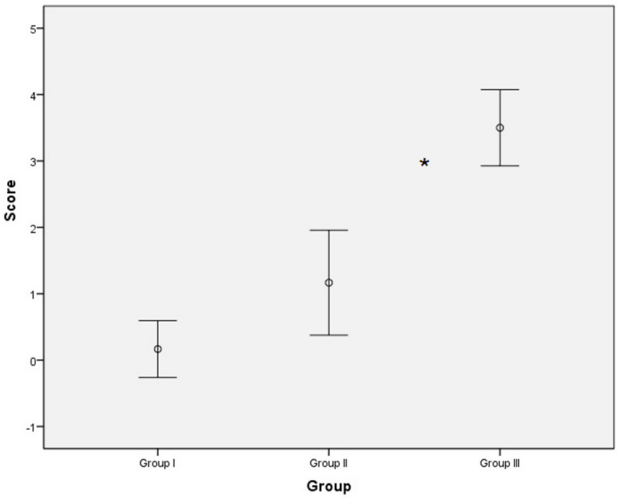


Figure 3. H&E and Prussian blue staining of lung tissues. Magnification: x100 or x200. Scale bar, 50 µm or 100 µm. a,d: sham, b,e: control, c,f: NAC pretreated rat lung tissue specimens.



Discussion

Ischemia/reperfusion injury is a common situation in both cardiac and peripheral arterial surgical procedures. The main injury occurs during the reperfusion period of the ischemic tissue in I/R and leads to cellular damage and organ dysfunction ultimately [17]. Remote organ damage is

another consequence of I/R injury. Cellular hypoxia results in decreased ATP production disrupts ion pump function and a shift to anaerobic glycolysis for energy production ensues. Activation of ROS is initiated during ischemia and during reperfusion period, a burst of ROS generation occurs which ultimately results in the respiratory burst of superoxide production, and thus massive oxidative stress is intensified that directly damages cells and induces both the programmed cell death responses, apoptosis and necroptosis and also a non-apoptotic cell death known as ferroptosis [18-20].

In this study, we aimed to show the effect of NAC pretreatment on the prevention of ferroptosis mediated lung injury induced by lower limb ischemia and reperfusion in a rat model. The present research found typical ferroptotic biochemical changes in control group animals as GSH depletion with increased tissue MDA levels meaning increased lipid peroxidation. However, NAC pretreatment is reported to be protective against remote organ injury induced through lower extremity I/R. NAC is reported to be targeting oxidized lipids [21]. Tissue MDA levels were significantly reduced in NAC pretreated rats meaning a reduced lipid peroxidation and ferroptosis. NAC is known to be a glutathione precursor and also a free radical scavenger that it exerts a direct antioxidant action through this property [22]. An important rate-limiting precursor of cellular GSH synthesis is cysteine and NAC exerts its indirect anti-oxidant action by de-acetylation to cysteine thus enhances the glutathione-S-transferase activity and supplies GSH for glutathione peroxidase-catalyzed detoxification of peroxides. GSH depletion is suggested to lead to an iron-dependent accumulation of ROS, which can cause cell death [3]. Our results suggested that tissue levels of GSH significantly increased in the NAC pretreated rats indicating a reduction in ferroptosis. NAC also exhibits anti-inflammatory properties through inhibiting the activation of NF-κB to prevent the release of pro-inflammatory cytokines and thus lead to less ROS production [12]. We used a NAC dose of 150 mg/kg body weight through the intraperitoneal route. The NAC dose was lower than those used in the literature, who reported that higher doses (300 mg/kg body weight) of NAC before ischemia improved liver and lung functions more efficiently than did lower doses (150 mg/kg body weight) [23,24]. We used the NAC dose that is mainly the preferred dose in the prevention of contrast-induced nephrotoxicity. We noted that administering NAC before lower limb I/R even in lower doses protected against remote organ injury in the lungs. The rats treated with NAC had significantly lower MDA levels, higher GSH levels and less neutrophil sequestration in the lungs after IR injury than the untreated rats.

Furthermore, we evaluated H&E staining and histopathological scores and lung wet-dry weight ratio. The results revealed the evidence of lung injuries such as accumulation of neutrophils, atelectasis, thickening of alveolar inter wall and alveolar edema in control group rats. Iron accumulation in the lungs was observed in Prussian Blue staining of lung tissue in control group animals revealing iron-dependent cellular damage, namely ferroptosis. However, the histopathological scores were significantly lower in NAC pretreated animals and additionally the amount of stainable iron was significantly reduced in NAC pretreated rats. NAC has been reported to be able to chelate metals, including iron [21]. Although we did not analyze the iron levels in the present study, we could able to show that the amount of stainable iron in lung tissues of control animals was significantly higher than NAC pretreated animals.

Our study has several limitations. First, we did or could not measure the tissue or blood levels of iron that has the main role in ferroptosis. Second, we did not compare the different doses of NAC; however, the NAC dose used in the present study indicated a significant protective effect when compared to the control group and this dose regimen has no restriction for the use in clinical settings.

Conclusion

Pretreatment with NAC potentially protects against injuries to remote organs, such as the lungs. NAC pretreatment improved edema, attenuated inflammation, and reduced lung injury severity in our lower extremity I/R rat model. These data are of clinical value, particularly given the common use, common dose and relative safety of NAC.

Declaration of conflict of interest

The authors received no financial support for the research and/or authorship of this article. There is no conflict of interest.

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