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Original Article

N-acetylcysteine reduces oxidative stress on end-organs in an ischemia-reperfusion rat model

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Abstract

Aim: In this study, we researched the effect of N-acetylcysteine (NAC) on parenchymal organs in ischemia-reperfusion injury in the rat models.

Material and Methods: Experimental animals divided into four groups: Sham group (Sh, n=6), Sham+NAC group (Sh+NAC, n=6), ischemia-reperfusion group (I/R, n=10) and N-acetylcysteine-given study group (I/R+NAC, n=10). For the Sham group and Sham + NAC group, only aortic exploration was performed. I/R group's abdominal aortas were clamped just inferior to the renal artery for 4 hours following. Then, the reperfusion period was allowed for one hour. I/R+NAC group received the same procedure as I/R group and additionally treated with NAC intraperitoneally. After these procedures, all rats were sacrificed. The parenchymal organs were excised for biochemical, flow cytometric and histopathologic examination. The data were evaluated statistically.

Results: In the I/R+NAC group, the MDA values in AC tissue were significantly lower than the I/R group (p:0.0032). MDA value in kidney tissue was significantly higher in the I/R group compared to the control groups (p:0.003). In the I/R+NAC group, the MDA value was significantly lower than the I / R group (p:0.0002). MPO values in lung tissue were found lower in the I/R+NAC group compared to the I/R group, it was not statistically significant (p:0.4497). In the I/R+NAC group, it was found statistically significantly lower than both the control and I/R groups in Flow cytometric evaluation (p:0.0002). In histopathological evaluation, the leukocyte count observed significantly higher in the I/R+NAC group compared to the control groups and was statistically significantly lower than the I/R group in histopathological evaluation (p:0,0004).

Conclusion: NAC reduces ischemia and reperfusion injury in parenchymal organs and especially in the lungs in a rat model.

Keywords: Anti-inflammatory; antioxidants; ischemia/reperfusion; N-acetylcysteine

INTRODUCTION

Local and systemic tissue damage caused by ischemia-reperfusion (I/R) syndrome, which may occur after arterial occlusion, is an important problem in vascular surgery (1). Even after successful revascularization, catastrophic conditions can be encountered, which can lead to dysfunction of the lower limbs, even requiring amputation, sometimes leading to death (2).

In recent years, advances in operative techniques have made important advances in rescuing ischemic extremities. Despite all this, studies conducted by many researchers on I/R in various organs have been shown to further increase both local and distant tissue damage, which occur after the ischemia period (3-11). This event, which we call reperfusion injury, occurs as a result of free oxygen radicals, neutrophil activation, tissue infiltration, and reduced microvascular circulation (12).

The common aim of many studies to prevent I/R damage after arterial occlusion is to preserve the integrity of the limb as well as to reduce or eliminate systemic effects that may occur. Research is ongoing to prevent unwanted reperfusion damage with the use of antioxidant substances (13-18).

In this study, we wanted to investigate the effectiveness of N-acetylcysteine (NAC) in experimental rats by reducing the reperfusion damage that may occur in distant organs prove this effect, we measured the end product of events that started with cell death and free oxygen radicals, malondialdehyde (MDA) and myeloperoxidase (MPO) in the lungs, liver, heart, and kidneys as markers of neutrophil activation. Also, we performed a quantitative evaluation of neutrophil infiltration in the lungs by flow cytometric (FCM) analysis. We tried to support the research by examining histopathological changes in the parenchymal organs.

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MATERIAL and METHODS

This experimental study was carried out in Karadeniz Technical University (KTU) Medical Faculty laboratories after the approval of the Local Ethics Committee for Animal Experiments. In this study, 32 male rats of Wistar-Albino type, whose weights varied between 250-350 grams, which were grown in KTU Surgical Research and Application Laboratories, were used. Experimental animals divided into four groups: Sham group (Sh, n=6), Sham+NAC group (Sh+NAC, n=6), ischemia-reperfusion group (I/R, n=10) and N-acetylcysteine-given study group (I/R+NAC, n=10).

In order to continue the anesthesia of rats using ether by inhalation for induction, intraperitoneal ketamine hydrochloride (Ketas®, Parke-Dawis, 50 mg/ml vial) was applied to 50 mg/kg. During the procedure, the breaths of the rats were continued spontaneously. Abdominal aortic exploration was achieved by performing median laparotomy above and below the navel following abdominal cleaning. Systemic heparinization (100 IU/kg) was performed. The Sh and Sh+NAC group rats without I/R were abdominally closed only after laparotomy and aortic exploration. In the I/R and I/R+NAC group rats with I/R formed, the abdominal aorta was explored and aorta was added from the infrarenal level with a bulldog clamp. Then the abdomen was closed. At the end of the four-hour ischemia period, the abdomen was reopened and occlusion in the aorta was removed. The abdomen was closed again to wait for an hour of reperfusion. In groups given NAC, 150 mg/kg NAC (Asist®, Hüsnü Arsan İlaçları A.Ş., 300 mg/3 ml ampoule) was administered intraperitoneally before these procedures. At the end of the experimental period, all rats were sacrificed by cardiac exsanguination. Samples were taken from the parenchymal organs of the subjects such as the lung, heart, liver, and kidney.

Histopathological examination

Tissue samples were detected in 10% formalin. Paraffin blocks were prepared after tissue follow-up. Serial sections of 5-micron thickness were made and stained with hematoxylin-eosin. In the lung samples, areas with excessive connective tissue except bronchi and capillary were excluded from the examination. Neutrophils in the alveolar and interstitium were counted at ten large magnifications (X40). Congestion and interstitial edema in lung tissue were evaluated semiquantitatively according to Tassiopoulos et al (19). Accordingly, for edema: 0 points; no edema, 1+; focal mild edema, 2+; focal intense edema, 3+; widespread edema; For congestion: 0 points; no congestion, 1+; focal mild congestion, 2+ focal intense congestion, 3+; scored as widespread congestion.

Kidney, liver and heart samples were evaluated in all fields with a light microscope. Klausner criteria were used in the study (20): Stage 0: normal kidney appearance, Stage 1: less than 5% necrosis in all sites, Stage 2: 5-25% necrosis in all sites, Stage 3: 25-75% necrosis in all sites, Stage 4: all More than 75% necrosis in the field. Cell swelling, vacuolization, congestion, and infiltration were also evaluated.

Flow cytometric examination

Since neutrophil infiltration will be studied in the lung tissue, fresh tissue samples were used. Since flow cytometry analysis will be made from suspended cells, the cell suspension was prepared by treating with fresh tissue mechanical disintegration method. An amount of 250-300 µl of the prepared cell suspension was added to the tubes where 20 µl of CD 177 monoclonal antibodies specific for neutrophils were placed and markings were made. Tubes were counted by passing the double laser Coulter Epics Elite ESP FCM device. Their positivity was determined as %. After counting the cells in the tubes, they were analyzed with 488 nm argon-ion laser at 15 mW. Cell flow rate was adjusted according to the cell density in the sample. Cells stained in FCM were rapidly passed through the dense laser field, while the cell-bound fluorochrome was activated by laser light. The fluorescent emission of the dye was determined and amplified by the sensitive phototube and transferred to the FCM computer.

Biochemical parameters

Malondialdehyde (MDA)

Tissue lipid peroxidation measurement was determined by the method of determining the MDA concentration as a result of the colorimetric reaction with thiobarbituric acid specified by Buege and Aust (21). When calculating, the coefficients of variation were taken as 5.2% for kidney, 4.8% for lung, 5.1% for liver and 5.3% for the heart. The protein concentration of the material was determined according to the Lowry method. The MDA concentration was unitized as nmol/mg protein.

Myeloperoxidase (MPO)

Schierwagen et al. Described the determination of tissue MPO activity by a modified method (22). While calculating, the coefficients of change (n = 5) were taken as 3.9% for kidney, 4.3% for lung, 5.0% for liver and 5.2% for the heart. The protein concentration of the material was determined according to the Lowry method. MPO activity was unitized as U/mg protein.

Statistical analysis

Biochemical, flow cytometric and histopathological parameters were used to evaluate the data of our study. Kruskal Wallis Variance analysis was used in the comparison of the four groups in the evaluation of neutrophil infiltration in the lungs whose flow will be examined cytometrically and in the determination of myeloperoxidase activity and malondialdehyde concentration in the tissues to be evaluated biochemically. The chi-square test was used for histopathological qualitative variables in the cells to be examined as a result of histopathological examination.

RESULTS

The experiment was completed smoothly. There was no mortality during the experiment. In statistical used Kuruskal Wallis Variant Analysis $p<0.05$; In the Mann-Witney U test used in pairwise comparisons between groups, $p <0.016$ was considered significant.

Table 1. Malondialdehyde (MDA) levels in tissues of the organs (nmol/mg tissue) of the groups

	Sh group (n:6)	Sh+NAC group (n:6)	I/R group (n:10)	I/R+NAC group (n:10)	p
Lung (MDA)	0.3±0.07	0.3±0.09	0.4±1.19	0.24±0.06	0.0056
Liver (MDA)	0.4±0.11	0.39±0.07	0.3±0.15	0.26±0.05	0.0012
Renal (MDA)	0.3±0.04	0.3±0.07	0.4±0.04	0.27±0.05	0.0002
Heart (MDA)	0.4±0.04	0.4±0.06	0.3±0.07	0.31±0.1	0.2358

The values are expressed as mean ± SEM (Standard Error of Mean) for each group. Sh: Sham group; Sh+NAC: Sham+NAC group; I/R: Ischemia/Reperfusion group; I/R+NAC: Ischemia/Reperfusion+NAC group. Statistical significance, p<0.05

MDA

MDA values in lung tissue were higher in the I/R group compared to the control groups, but this height was not statistically significant (p:0.8). In the I/R+NAC group, the MDA value was significantly lower than the I/R group (p:0.0032). In addition, the MDA value in the I/R+NAC group was found to be lower than the control groups' values, but this decrease was not statistically significant (p: 0.02). MDA value in KC tissue was found in the highest control groups. There was a significant decrease in MDA value in the I/R group compared to the control groups (p:0.0143). There was also a low MDA value in the I/R+NAC group compared to the I/R group, but it was not statistically significant (p:0.0191). MDA value was significantly lower in the NAC group compared to the control groups (p: 0.022). MDA value in kidney tissue was significantly higher in the I/R group compared to the control groups (p:0.003). In the I/R+NAC group, the MDA value was significantly lower than the I / R group (p:0.0002). There was no significant difference between the control groups and the I/R+NAC group (p:0.2703). MDA values in the heart tissue were not statistically significant between the groups (Table 1).

MPO

MPO value in lung tissue was found to be significantly higher in the I/R group compared to the control groups (p:0.0048). Although it was found lower in the I/R+NAC group compared to the I/R group, it was not statistically significant (p:0.4497). There was no significant difference between the control groups and the I/R+NAC group (p:0.022). MPO value in liver tissue was found to be significantly higher in the I/R group compared to the control groups (p:0.0084). In the I/R+NAC group, the values were found to be significantly lower and close to the control groups compared to the I/R group. MPO value in kidney tissue; It was found to be significantly higher in the I/R group compared to the control groups (p:0.0022), but significantly lower in the I/R+NAC group (p:0.9097). In the I/R+NAC group, MPO values were also found significantly higher than the control groups (p:0.0022). There were no statistically significant MPO values between the groups in the heart tissue (Table 2).

Table 2. Myeloperoxidase (MPO) levels in tissues of the organs (U/mg tissue) of the groups

	Sh group	Sh+NAC group	I/R group	I/R+NAC group	p
Lung (MPO)	1.3±0.2	1.3±0.09	4.5±2.1	3.7±1.24	0.0042
Liver (MPO)	0.12±0.03	0.11±0.02	0.3±0.23	0.08±0.05	0.0007
Renal (MPO)	0.01±0.02	0.01±0.07	0.09±0.04	0.09±0.04	0.0031
Heart (MPO)	5.2±1.5	5.1±0.09	2.4±1.95	2.4±3.01	0.0617

The values are expressed as mean ± SEM (Standard Error of Mean) for each group. Sh: Sham group; Sh+NAC: Sham+NAC group; I/R: Ischemia/Reperfusion group; I/R+NAC: Ischemia/Reperfusion+NAC group. Statistical significance, p<0.05

Flow cytometric evaluation

The amount of neutrophil infiltration of the lungs in % of flow cytometrically. In the I/R+NAC group, it was found statistically significantly lower than both the control and I/R groups (p:0.0002) (Table 3).

Histopathological evaluation

In the histopathological evaluation of the lungs, the

leukocyte counts in the ten areas at major magnification were significantly higher in the I/R group compared to the control groups (p:0.022). The leukocyte count observed significantly higher in the I/R+NAC group compared to the control groups and was statistically significantly lower than the I/R group (p:0,0004). The leukocyte counts remained significantly higher in the I/R+NAC group compared to the control groups (p:0,0071). The evaluation of the histopathological sections of the kidney samples of the groups was made according to the criteria of Klausner et al (19). In addition, cell swelling was examined according to vacuolization, congestion and infiltration conditions. However, no statistical difference was observed between the groups. The same is true for liver and heart tissue samples (Table 3).

Table 3. Flow cytometric and histopathologic examination in tissues of the lung. The positivity of the flow cytometric evaluation was determined as %. Neutrophil counts in the alveolar and interstitium were counted at ten large magnifications (X40) in histopathologic evaluation

	Sh group	Sh+NAC group	I/R group	I/R+NAC group	p
FCM %	14.2±2.3	13.8±2.7	14.4±1.8	5.6±1.2	0.0002
Histopathologic examination	71.2±11.6	72.3±0.9	289.1±49.1	153.1±53.2	0.0001

The values are expressed as mean ± SEM (Standard Error of Mean). Sh: Sham group; Sh+NAC: Sham+NAC group; I/R: Ischemia/Reperfusion group; I/R+NAC: Ischemia/Reperfusion+NAC group. Statistical significance, p<0.05

DISCUSSION

Ischemia-reperfusion injury after revascularization therapy can cause unintended results. Although reperfusion is a vital event for ischemic tissue, it can lead to the production of free oxygen radicals, such as superoxide, hydroxyl radical, and hydrogen peroxide. Free radicals can disrupt antioxidant systems and lead to irreversible cell damage and organ dysfunction (1,2,11,15-18).

In this study, we investigated the role of NAC in reducing reperfusion injury by creating experimental I/R syndrome in the abdominal aorta by biochemical and histopathological evaluations. As a result of the experiment, in the I/R+NAC group, MDA levels as the biochemical parameter and leukocyte counts in FCM analysis were found to be significantly lower than the I/R group for lung tissue. Although similar biochemical changes have been shown for liver and kidney tissues, no statistically significant improvements in heart tissue have been observed. Histopathological examination in light microscope showed that the number of leukocytes in the lungs decreased significantly in the I/R+NAC group compared to the I/R group, but the same effect was not observed in the examination of other organs. We think that this is the different response of the organs to the I/R periods.

As a result of many studies, it has been shown that the damage continues not only in ischemia but also in the reperfusion period (3-11). During ischemia, the cell is unable to maintain biomembrane integrity. Increased biomembrane damage leads to the release of polyacid fatty acids and the formation of fatty acid radicals. At this stage, when oxygen enters the environment again, fatty acid radicals combine with oxygen to form a lipid peroxidation reaction. MDA is a product of this reaction and is a suitable marker for showing the level of oxidative damage (2). Cells that play the leading role in cellular injury in I/R damage are leukocyte. MPO activity is an important parameter to determine the effectiveness of leukocyte (2).

Many operative techniques and drugs are still being tried to minimize damage in I/R syndrome. Recently, NAC has been popularly researched in this area. NAC has found various clinical uses due to its many features (1-6). Thanks to the sulfhydryl in its structure, it is widely used mucoid-secretolytic. Due to its thiol group content, fulminant triggered by acetaminophen poisoning has been used successfully in hepatic insufficiency. Due to its anti-nephrotoxic feature, it has a use in radiographic contrast agent nephrotoxicity. It also has antianginal effects with its features such as vasodilation, increasing nitrate levels, stabilization of atherosclerotic processes and modulation of arrhythmogenesis. In addition, NAC is a direct protective effect on free oxygen radicals due to its effects on the cysteine-cysteine redox mechanism and its connections with glutathione. It is also indirectly effective on free oxygen radicals by disrupting the -SH groups of enzymes and peptides in membranes (23).

There are many studies on NAC and I/R injuries. In these studies, it was emphasized that NAC has a reducing effect on I/R damage. However, most of these studies were performed in isolated organ I/R injuries. The number of studies on indirectly affecting the parenchymal organs in ischemia and reperfusion of the infrarenal abdominal aorta is very few. Our study with NAC to reduce distant organ damage in I/R syndrome is a candidate for being one of the leading studies in the literature.

The lung is one of the most important target organs that affect mortality and morbidity in I/R syndromes (1,5,6,17,19). In our study, when we investigated the

change of biochemically, FCM and histopathological evaluations between the groups in lung tissue, positive effects were observed in the drug group. These findings were consistent with the literature. In order to make these evaluations more meaningful statistically, we believe that new studies should be conducted with larger series, especially where reperfusion times are kept longer.

In I/R syndrome, the kidneys are among the affected parenchymal organs, such as the lungs (4,13-15,20). In our study, the positive effects of NAC on reperfusion damage in the kidneys were once again proven. The liver in I/R damage can be said to be a relatively more resistant organ. Our study has proven once again that NAC is one of the key drugs in reducing liver damage. We did not find that NAC was statistically significant in correcting heart damage during the experiment.

CONCLUSION

NAC, which has clinically different uses, has positive effects in reducing I/R damage. This effect was seen more clearly in the lung and kidney tissues. However, this experimental model can be developed and enriched. In light of these studies, I/R damage may become more important in the clinical use of NAC.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study was approved by the Local Ethics Committee for Animal Experiments of Karadeniz Technical University and conducted in compliance with the institutional and international guidelines were followed for the care and use of laboratory animals.

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