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ORIGINAL ARTICLE

Protective Effects of Tarantula Cubensis Extract (Theranecron) and N-acetylcysteine on the Liver in an Experimental Model of Ischemia-Reperfusion Injury in Rats

Sıçanlarda Deneysel Bir İskemi Reperfüzyon Hasarı Modelinde Tarantula Cubensis Ekstresi (Theranecron) ve N-asetilsisteinin Karaciğer Üzerindeki Koruyucu Etkileri

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Abstract

Introduction: In this study, we aimed to show the protective effects of N-acetylcysteine (NAC) and Tarantula cubensis extract (TCE) against ischemia-reperfusion (IR) damage on the liver.

Methods: The rats for which an IR injury model was created experimentally were divided into five groups with seven in each group. Only laparotomy was performed in the control group. The hepatic hilus was clamped in other groups, and 60 min of ischemia was performed. Then, the clamp was opened, and 6 h of reperfusion was achieved. IR model was applied to the sham group. The NAC group was given 50 mg/kg NAC intraperitoneally (IP) 60 min before ischemia. The TCE group was given 10 mg/kg TCE as IP. In the NAC + TCE group, these drugs were given in combination at the same dose.

Results: Liver function test values were higher in the treated groups compared with the control group and the difference was significant ($p<0.05$). When the gamma glutamyl transferase levels were compared, the levels in the sham group were statistically significantly higher than the other groups ($p<0.01$). When antioxidant enzyme levels were compared, it was observed that the sham group was significantly lower than the other groups ($p<0.01$).

Discussion and Conclusion: We conclude that when TCE is used alone or in combination with NAC, it protects the liver against the negative effects of IR.

Keywords: Ischemia; Liver; N-acetylcysteine; Reperfusion; Tarantula cubensis extract

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Ischemia is defined as the decrease in blood flow as a result of occlusion of the vessels feeding the tissue. The decrease of oxygen in the cell activates anaerobic metabolism, and the free oxygen radicals formed cause organ failure.[1] In liver surgeries performed for transplantation, liver resection for any reason, or injuries as a result of trauma, it may be necessary to temporarily interrupt the blood flow. For this purpose, the hepatic artery, portal vein, and common bile duct in the liver hilum are temporarily blocked. [2] If this blockage continues for a long time, ischemia and eventually cell death and organ failure occur.[3] When the liver (liver) is revascularized, reperfusion damage occurs additionally, which is the main cause of postoperative organ failure. Free oxygen radicals are the main responsible substances in ischemia-reperfusion (IR) damage. Antioxidant mechanisms protect the cell from harmful effects by inactivating these oxygen radicals. When this balance is disrupted, anaerobic metabolism activates and reveals IR damage by mechanisms including mitochondrial dysfunction, calcium overload, and activation of Kupffer cells and cytokines.[4] It is not possible to measure the level of free oxygen radicals due to their short half-life time. Therefore, malondialdehyde (MDA), the final product of lipid peroxidation, can be used to determine the effect of free radicals on cell membrane lipids. In addition, superoxide dismutase (SOD), a member of the antioxidant system, catalyzes the conversion of superoxide anion into hydrogen peroxide (H_2O_2) and oxygen (O_2) molecules. Catalase (CAT), on the other hand, acts as an antioxidant by taking part in the conversion of H_2O_2 to water and O_2 molecules.[5] Drugs that can correct the deterioration in microcirculation and inactivate free O_2 radicals should have therapeutic effects that will prevent organ failure, which may occur after surgery, by minimizing liver IR damage.

N-acetylcysteine (NAC) is an N-acetylated derivative of L-cysteine, a natural amino acid, and is a mucolytic agent. In paracetamol toxicity, the glutathione reserve in the body cannot conjugate the excessive accumulated N-acetyl-p-benzoquinone metabolite. It has been determined that NAC therapy protects the liver from toxic effects by replenishing its glutathione reserve. Hu et al.[6] showed that survival after liver transplantation improved in patients who received NAC. According to the recommendation of the American Association for the Study of Liver Diseases (Level I), it is stated that NAC treatment is beneficial in all drug-induced acute liver failure.

Theranecron, Tarantula cubensis extract (TCE), is obtained by alcohol extraction of spider venom. TCE is an anti-inflammatory, antitumor, anti-infective, regenerative, and resolitive agent. Although the mechanism of action of TCE is not known exactly, it has been stated that it creates a border around the necrotic tissue formed after surgery, prevents the movement of inflammatory structures and catabolic enzymes from the necrotic tissue to the environment, and prevents further deterioration of the connective tissue structure.[7,8]

In this study, we aimed to show and compare the protective efficacy of NAC,[9] which has been shown in studies on the protective effect on KC in IR damage, and TCE, which has not been studied before, separately and in combination against IR damage.

Materials and Methods

This experimental study was conducted by T.C. Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research and Application Center Experimental Animals Local Ethics Committee (DUHADEK) and was held in DUHADEK laboratory by obtaining the ethics committee approval dated June 3, 2020 and protocol number 2020/21. This study was conducted in accordance with the Declaration of Helsinki.

A total of 35 male Sprague–Dawley rats, each weighing 200–250 g, were used in the study. All groups were followed in separate cages with no food, water, and movement restrictions at 20–22°C, 50%–60% relative humidity, and 12-h day and night cycles. Five different groups were formed as control, sham, NAC, TCE, and NAC + TCE groups, each with 7 rats. TCE used in the study was supplied as Theranekron® (richter pharma, Erse Veterinary Pharmaceutical Warehouse, Istanbul) and NAC as Nacosel® (Haver, Istanbul).

The rats were kept fasting for 12 h before surgery, and were allowed to drink only water. Operations were performed under sterile conditions and anesthesia. For anesthesia, 40 mg/kg pentobarbital was administered intraperitoneally (IP), and after general anesthesia, the rats were placed in a dorsal reclining position and the anterior abdominal wall was shaved. Then 10% povidone-iodine (Isosol®, Central Lab. Ilac San. Turkey) and antisepsis were provided. A wide midline incision was made after anesthesia to apply the IR model. The liver hilum was exposed, and the hepatic artery, portal vein, and bile duct were closed with an atraumatic clamp to cause IR injury. Control group (n=7): laparotomy was performed without clamping the liver hilum. Sham group (n=7): vascular occlusion was achieved by clamping the liver hilum. NAC group (n=7): 50 mg/kg NAC was administered via IP 60 min before the liver hilum was clamped. TCE group (n=7): 60 min before the hepatic hilus was clamped, 10 mg/kg TCE IP was given. NAC + TCE

group (n=7): 10 mg/kg TCE + 50 mg/kg NAC was given IP 60 min before the liver hilum was clamped. Reperfusion was achieved by opening the clamp at the 60th minute in all groups with ischemia. As long as the abdomen was open, 10 mL/kg/h 0.9% isotonic sodium chloride IP was given to compensate for the fluid loss. After reperfusion, the incision was closed with 3/0 vicryl. All subjects were sacrificed by intracardiac blood aspiration 6 h after reperfusion.[9]

Blood samples were centrifuged in a biochemistry tube at 3000 rpm for 10 min, and serum was separated. In the serum obtained, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT) levels were measured using a modular autoanalyzer (Roche, Mannheim, Germany). Serum ALT, AST, and GGT activities were expressed in units per liter (U/L).

After the subjects were sacrificed, the liver was divided into two parts, and a part of it was fixed in 10% formaldehyde solution for 24 h for histopathological examination. It was stained with hematoxylin and eosin. Paraffin blocks were prepared, and sections with a thickness of 5 µm were prepared and examined under light microscopy. The scores of inflammation, necrosis, and degeneration in hepatic tissue were compared. Histological findings were graded as none (0), mild (1), moderate (2), or severe (3).

When preparing the liver homogenate, 1 g liver tissue was mixed with 9 mL normal saline on ice and centrifuged (4000 rpm, 10 min). Uchiyama and Mihara used the method for measuring MDA. In this method, 8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.8% thiobarbituric acid and n-butanol were used as reagents. MDA level was determined spectrophotometrically. Plasma and leukocytes of homogenate obtained from the tissue were removed by centrifugation for SOD measurement. The erythrocyte was washed twice with saline and hemolyzed with cold deionized water. The hemoglobin concentration of the homogenate was adjusted to 10 g/dL, and the SOD activity in the homogenate was measured spectrophotometrically. Aebi method was used for CAT measurement. CAT activity was measured spectrophotometrically in concentrated homogenate diluted with 1/1000 phosphate buffer obtained from the tissue.[10,11]

Statistical Analysis

Statistical analyses were performed using SPSS version 21 software. The data were expressed with a mean of 1 standard deviation and used as frequency (%) for continuous variables and qualitative data. Analysis of variance was performed using Tukey's post hoc test for comparisons between more than two groups. ANOVA test was used to test group differences in all groups studied. The 5% false discovery rate was controlled with the Benjamini–Hochberg p-value correction. The Chi-squared test was used for the analysis of binary variables. All tests were double-sided, and $p<0.05$ was considered statistically significant with a reliability above 95%.

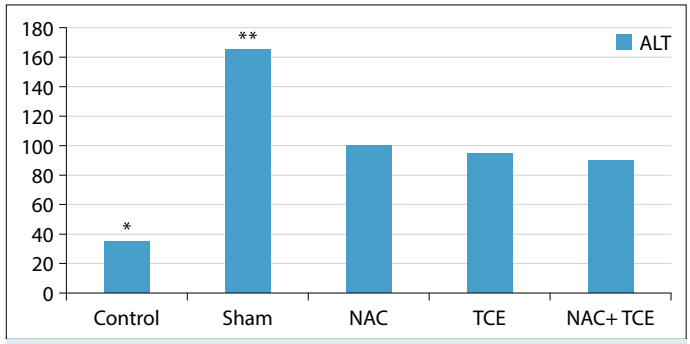

Figure 1. Serum ALT levels in groups *p<0.01 versus control group **p<0.01 versus sham group.

ALT: Alanine aminotransferase; NAC: N-acetylcysteine; TCE: Tarantula cubensis extract.

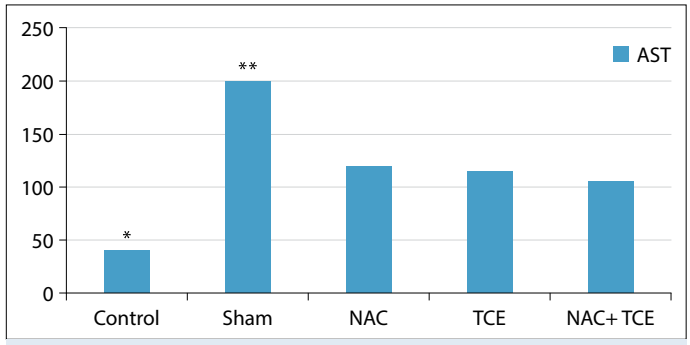

Figure 2. Serum AST levels in groups *p<0.01 versus control group **p<0.01 versus sham group.

AST: Aspartate aminotransferase; NAC: N-acetylcysteine; TCE: Tarantula cubensis extract.

Results

There was no loss of subjects during the study. ALT and AST levels were significantly higher in the sham group compared with the other groups ($p<0.01$). ALT and AST values were higher in the treated groups compared with the control group, and the difference was significant ($p<0.01$). ALT and AST values were less elevated in the treated groups in the NAC + TCE group, but this difference was not statistically significant when compared with the NAC and TCE groups. A comparison of ALT and AST levels between groups is shown in Figure 1 and 2. When the GGT levels between the groups were compared, the levels in the sham group were

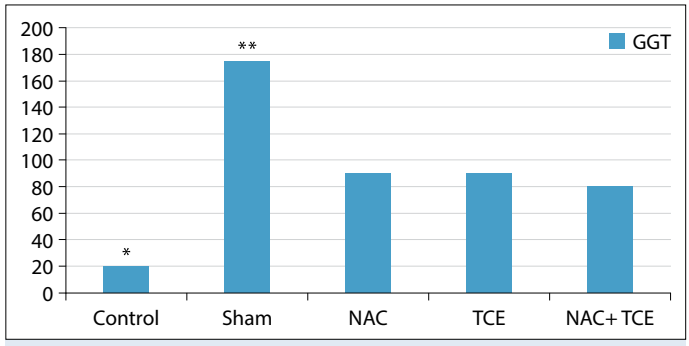

Figure 3. Serum GGT levels in groups *p<0.01 versus control group **p<0.01 versus sham group.

GGT: Gamma glutamyl transferase; NAC: N-acetylcysteine; TCE: Tarantula cubensis extract.

significantly higher than the other groups (p<0.01). GGT levels were significantly higher in the drug-administered groups compared with the control group (p<0.01). The difference in GGT levels between drug-administered groups was not significant. The least increase was seen in the NAC + TCE group compared with the control group. The intergroup GGT levels are shown in Figure 3.

When the levels of MDA, the final product of lipid peroxidation, were compared with the antioxidant system enzymes SOD and CAT after experimentally performed hepatic IR injury, the following results were obtained: MDA level was found to be significantly higher in the sham group compared with the other groups (p<0.01). When the control group and the groups that were given medication were compared, the MDA level was higher in these groups than in the control group, but the difference was not statistically significant. Differences between MDA levels in NAC, TCE, and NAC + TCE groups were not statistically significant. When SOD and CAT levels were compared, it was seen that the sham group was significantly lower than the other groups (p<0.01). SOD and CAT levels were highest in the drug-administered groups in the NAC + TCE group, and the difference was statistically significant compared with the NAC and TCE groups (p<0.01), but not significant compared with the control group. A comparison of MDA, SOD, and CAT levels between groups is shown in Table 1.

Degeneration, inflammation, and necrosis scores were compared between the groups in the histopathological examination of the liver tissue. Histopathological scores were significantly higher in the sham group compared with the control and treatment groups (p<0.01). When NAC and TCE were applied together, the liver protective effect was more pronounced histopathologically than in the groups in which they were applied separately (p<0.01). In the groups

Table 1. Effects of NAC and TCE on Hepatic levels of MDA and antioxidant enzymes in rats

Group	MDA (mg/dl)	SOD (mg/dl)	CAT (mg/dl)
Control	5.02±0.25	161.42±5.74 **	90.51±3.13**
Sham	7.30±0.55 *	82.15±12.41*	40.71±2.21*
NAC	5.10±0.15	135.24±6.33	60.22±2.31
TCE	5.08±0.20	138.63±7.12	58.96±2.42
NAC+TCE	5.06±0.11	164.25±4.62**	88.46±1.53**

Data are given as mean±standard deviation (n=7). *: P<0.01 vs. sham group; **: P<0.01 vs. NAC+TCE and Control groups; NAC: N-acetylcysteine; TCE: Tarantula cubensis extract; MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase.

where NAC and TCE were applied separately, histopathological scores were higher than the control and NAC + TCE groups, but lower than the sham groups. The histopathological data of the groups are shown in Table 2. Microscopic images of degeneration, inflammation, and necrosis seen after the experimental model with normal liver tissue are shown in Figures 4–7.

Discussion

Most of the complications that occur after liver surgery occur as a result of the pathological processes caused by IR injury. IR damage is the main cause of graft rejection and graft dysfunction after orthotopic liver transplantation. IR damage is the main cause of graft rejection and graft dysfunction after orthotopic liver transplantation. Prevention of IR damage is very important in terms of increasing the success rate of liver surgeries as well as preventing the loss of the donated organ.[12,13]

N-acetyl cysteine is an active substance that has a protective effect on the liver and is used for this purpose today. Mumtaz et al.[14] divided the patients with acute liver failure other than acetaminophen into two groups in their clinical study. In the group given NAC treatment, the rate of attachment to the mechanical ventilator, length of hospital stay, and mortality rates were found to be lower than those in the control group. Lauz Medeiros et al.[15] evaluated biochemical parameters and small intestine histopathology at different reperfusion times after liver ischemia. They found that ALT, AST, ALP, and LDH levels increased in correlation with the reperfusion time. They found that the AST value increased less than the other groups at the end of 6 h of reperfusion in the group given NAC and that NAC was morphologically protective on the small intestine after 30 min of reperfusion. In their experimental study, Su Lee et al.[16] divided the rats into two groups and applied IR + 30% hepatectomy. One of the groups applied 150 mg/kg NAC

Table 2. Expression of liver specimen parameters in relation with group

Hepatic tissue	Expression	Groups					χ^2	p
		Control	Sham	NAC	TCE	NAC+TCE		
Nekrosis	0 (0%)	7	0	0	0	0		
	1 (<25%)	0	0	4	3	6	25.632	<0.01
	2 (25-50%)	0	3	3	4	1		
	3 (>50%)	0	4	0	0	0		
Degeneration	0 (0%)	7	0	0	0	0		
	1 (<25%)	0	0	3	3	6	16.285	<0.01
	2 (25–50%)	0	3	3	3	1		
	3 (>50%)	0	4	1	1	0		
Inflammation	0 (0%)	7	0	0	0	1		
	1 (<25%)	0	0	3	3	6	12.357	<0.01
	2 (25–50%)	0	2	3	3	0		
	3 (>50%)	0	5	1	1	0		

NAC: N-acetylcysteine; TCE: Tarantula cubensis extract.

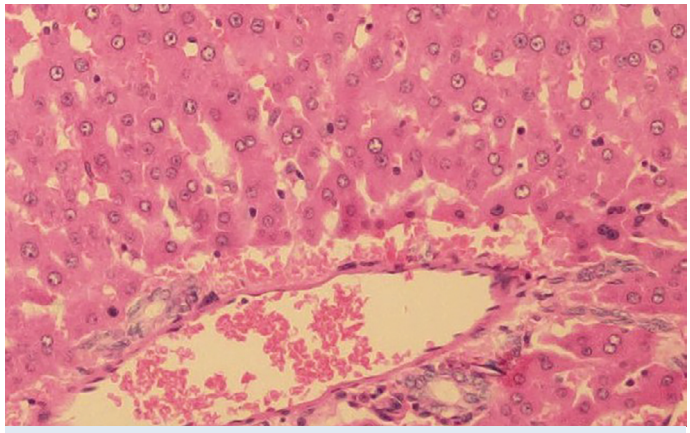

Figure 4. Normal liver tissue (H&E *200).

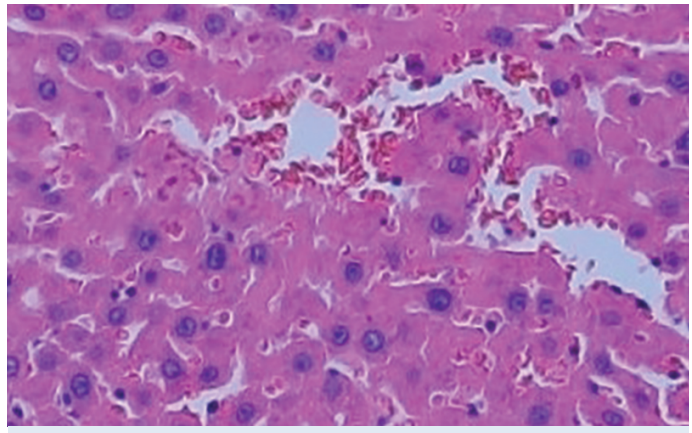

Figure 6. Necrosis (H&E *200).

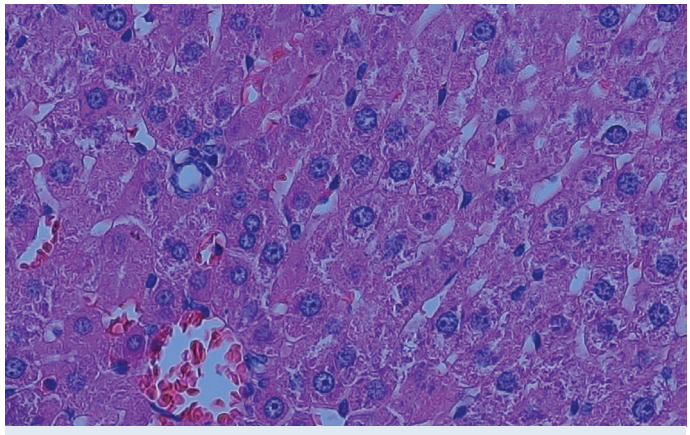

Figure 5. Degeneration (H&E*400).

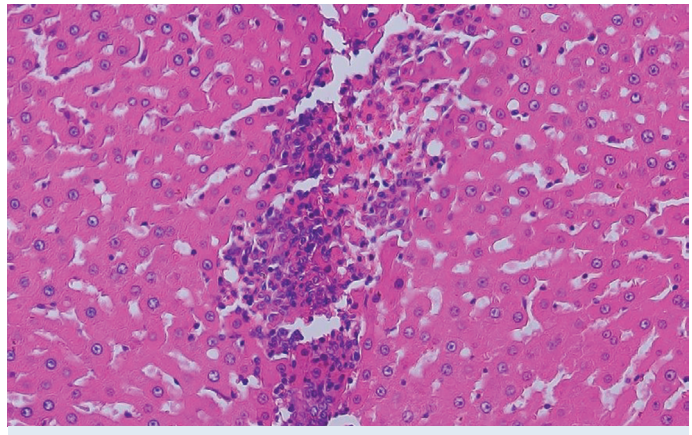

Figure 7. Inflamation (H&E *200).

treatment. ALT and AST values were found to be significantly lower in the group receiving NAC. They also showed histopathologically that NAC treatment was protective of the liver. Cayuela et al.[17] demonstrated the protective effect of NAC on the liver against IR damage by reducing oxidative stress, inflammatory response, and cell death in

rats with steatohepatitis. Considering the literature information, the low levels of MDA in the groups given NAC and TCE, high levels of antioxidant enzymes, lower increase of ALT, AST, and GGT levels in the treatment groups, and the protective effect of NAC and TCE in the histopathological evaluation of liver compatible.

Although there are a limited number of studies in the literature regarding TCE, anti-inflammatory and accelerating effects of wound healing have been shown in bovine cutaneous papillomatosis, canine mammary adenocarcinoma, and injuries created by an experimental model in rats.[8,18,19] In their study, Kızılay et al.[20] investigated the effect of TCE in the damage model created in the rat sciatic nerve. As a result of the study, they stated that TCE reduces axonal and myelin damage and this neuroprotective effect can be demonstrated by suppressing proinflammatory cytokines such as TNF-α, IL-1, and IL-6.

Oryan et al.[21] created a model of damage to the rabbit hindfoot tendon in their experimental study. They applied 1 µg/kg TCE to the injured area on days 3, 7, and 10 for treatment. They showed that TCE has positive structural and biomechanical effects on tendon healing. These results are consistent with our study, with its anti-inflammatory activity.

Tanyeli et al.[22] investigated the protective effect of TCE on the lungs as a result of sepsis caused by cecum perforation in rats. They found that the TNF-α level was significantly lower in the group receiving TCE treatment. They found that it increased the total antioxidant capacity. In the histopathological examination of the lung, alveolar occlusion was found to be less in the group in which low-dose TCE was administered, but edema and inflammation were evident, while normal findings were observed in the group given high-dose TCE. These findings are consistent with our study in that it increases antioxidant system enzymes and has a protective effect on the organ histopathologically.

Lipid peroxidation products created by free oxygen radicals cause damage to tissues with protein denaturation. These products can also trigger a systemic inflammatory response. Inflammatory mediators that occur cause organ failure.[23] Karabacak et al.[24] demonstrated the protective effect of TCE on liver damage due to aflatoxin with its anti-inflammatory effect. In another study conducted on cows with papilledema, given TCE, it was shown that the total antioxidant capacity increased and the total oxidant capacity decreased after 15 days.[25] All this literature information shows the anti-inflammatory efficacy of TCE and is consistent with our study.

There are studies with different active substances in the literature to prevent hepatic IR injury. Carnosine, indigo carmine, sodium nitrite, and ursodeoxycholyl lysophosphatidylethanolamide are some of these active ingredients. Each active ingredient had a more or less protective effect on the liver. However, currently, very few drugs are used in clinical practice.[13,26–28] Studies will continue to investigate the active ingredient that will prevent IR damage to increase the success rate in liver surgery.

Conclusions

IR injury is common in clinical practice after liver surgery. This damage can cause organ failure and mortality. Therefore, drugs that will prevent IR damage will be life-saving. This study is the first to show the liver protection of TCE against hepatic IR damage. As a result, we can say that TCE against IR is as effective as NAC when used alone and protects the liver more against IR damage when used with NAC.

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Authorship Contributions: Concept: ED, AT; Design: NBA, SK; Supervision: AÇ, ED; Materials: AÇ, SK; Data Collection or Processing: NBA, AT; Analysis or Interpretation: SK, AT; Literature Search: ED, AÇ; Writing: ED, AÇ; Critical Review: NBA, AT.

Conflict of Interest: No conflict of interest between the authors.

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