# The effects of N-acetylcysteine on intestinal ischemia/reperfusion injury in rats

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## ABSTRACT

الأهداف: تقييم آثار عقار إن–أسيتيلسيستين (NAC) على إصابة نقص التروية / إعادة الانصباب للمصران لدى الجرذان.

الطريقة: تم تقييم عدد 48 جرذ من نوع ويستار– ألبينو إلى ست مجموعات: مجموعة التحكم (C)، المجموعة المصابة بنقص التروية بالدم (I)، المجموعة المصابة بنقص التروية–إعادة الانصباب (IR)، المجموعة المصابة بنقص التروية للدم وتلقت عقار إن– أسيتيلسيستين (IN)، المجموعة المصابة بنقص التروية– إعادة الانصباب + تلقت عقار إن–أسيتيلسيستين (IRN)، المجموعة المصابة بإعادة الانصباب+تلقت عقار إن–أسيتيلسيستين (RN). أجري الفحص المرضي النسيجي لجميع المجموعات. تم تقييم مستويات الأنسجة والبلازما وعينات الكريات الحمراء ومستويات مالونديالديهيد (MDA)، فوق التأكسد (SOD)، جلوتاثيون (GSH)، وأكسيد النتريك (NO). أجريت هذه الدراسة الحالية بجامعة تراكيا واسطنبول – تركيا، خلال الفترة ما بين ديسمبر 2002م وحتى يوليو 2003م.

النتائج: تبين وجود تلف نسيجي مرضي شديد لدى مجموعة نقص التروية بالدم وإعادة الانصباب للمصران، وتمت مراقبة هذا التلف لتخفيف جرعة عقار (NAC). تبين وجود أقل مستوى للبلازما (MDA) لدى مجموعة (RN)، كما تبين أن مستويات النسيج (GSH) مرتفعة لدى مجموعة (RN) أكثر من مجموعة (IRN).

خاتمة: تبين أن لدى المعالجة بعقار إن–أسيتيلسيستين (NAC) آثار مهمة على إصابة نقص التروية بالدم للمصران كذلك إعادة الانصباب لدى الجرذان. تسبب المعالجة بعقار إن–أسيتيلسيستين (NAC) تحسناً في النتائج النسيجية المرضية لتلف نقص التروية / إعادة الانصباب (I/R). تحمي المعالجة بعقار إن–أسيتيلسيستين (NAC) الأنزيمات المضادة للأكسدة في الأنسجة والبلازما والكريات الحمراء والتي تعتبر مهمة في إصابة نقص التروية/إعادة الانصباب (I/R) لدى الجرذان.

**Objectives:** To evaluate the effects of N-acetylcysteine (NAC) on the injury of intestinal ischemia-reperfusion.

**Methods:** Forty-eight Wistar-Albino rats were divided into 6 groups: as control, ischemia, ischemia-reperfusion, ischemia + N-acetylcysteine, ischemia-reperfusion + N-acetylcysteine (IRN), and reperfusion + N-acetylcysteine (RN). Histopathologic examination was performed to all groups. In the tissue and plasma, and erythrocyte samples, malondialdehyde, superoxide dismutase, glutathione, and nitric oxide (NO) levels were evaluated. The present study was carried out in Trakya and Istanbul University, Edirne, Turkey between December 2002 and July 2003.

**Results:** The most severe histopathological damage was seen in the intestinal ischemia-reperfusion group, and this damage was observed to be reduced by NAC administration. Lowest plasma malondialdehyde levels were observed in RN group. The tissue glutathione levels were found to be higher in RN group than those in IRN group.

**Conclusion:** It was found that administration of NAC has important effects on the injury of intestinal ischemia, as well as, reperfusion in rats. N-acetylcysteine administration causes an improvement in the histopathologic findings of ischemia/reperfusion damages. The N-acetylcysteine treatment protects the antioxidant enzymes in the tissue, plasma, and the erythrocytes, which are crucially important in the intestinal schemia/reperfusion injury in rats.

Ischemia/reperfusion (I/R) injury is the most common clinical entity in surgical practice. It may occur secondary to pathologies such as intussception, intestinal volvulus, incarcerated hernia, fibrous adhesions of the intestine, and also mesenteric artery occlusion. Oxidative stress develops when tissues are deprived of oxygen and other metabolic compounds. This activates both certain local and systemic mechanisms. Nevertheless, serious clinical syndromes, such as multiple organ failure may develop with the activation of a series of oxidative stress systems for which the free oxygen radicals are responsible.[1] N-acetylcysteine (NAC) is a sulfhydryl group transmitter, and also a strong antioxidant as a free radical scavenger of the thiol groups.[2] Since NAC, a precursory of glutathione (GSH), is capable of detoxifying free radicals and reactive electrophiles, clinical use of this agent was broadened within years.[3] Reactive electrophiles are molecule particles which form new radicals, and are highly attractive for electrons. N-acetylcysteine also reduces hydrogen peroxide ($H_2O_2$) levels and thus, protects the cells against toxic effects of $H_2O_2$. The protective effect of NAC against toxic radicals also include the increase of GSH biosynthesis, since it is a GSH precursor.[4] All these data generate the idea, to investigate the antioxidant effects of NAC on both intestinal ischemia, and reperfusion injury. In this study, we evaluate different types of injuries in rats and the effects of NAC on each injury, as well as, NAC's probable protective effects over such injuries.

**Methods.** The present study was carried out at Trakya and Istanbul University, Turkey between December 2002 and July 2003. The Ethical Committee of Trakya University approved all animal procedures, and the experimental protocol. Forty eight adult Wistar-Albino rats, weighing 380-450g, were included in this study. Rats were provided by the Experimental Research Center of the Medical Faculty of Trakya University. The rats were kept in a windowless animal quarter where temperature ($22 \pm 2°C$), and illumination were (light on at 7 am, and off at 9 pm: 14 hours light/10 hours dark cycle) automatically controlled. Humidity ranged from 50-55%. The rats were fed with standard rat chow and tap water ad libitum. The rats were randomly divided into 6 groups (n=8 each) as follows: 1) control: sham control, 2) ischemia: ischemia was performed for 45 minutes, 3) ischemia-reperfusion (IR): ischemia (45 minutes), and reperfusion (60 minutes) practices were performed, 4) ischemia+N-acetylcysteine (IN): 100 mg/kg of NAC (Asist®, Bilim-Istanbul) was administered from the tail vein at the beginning of ischemia, 5) ischemia-reperfusion+N-acetylcysteine (IRN): 100 mg/kg of NAC was administered from the tail vein at the beginning of ischemia, and 6)

reperfusion+N-acetylcysteine (RN): with the start of reperfusion just after the ischemia (45 minutes), 100 mg/kg of NAC was administered from the tail vein. Before the surgery, 5-10 mg/kg xylazine hydrochlorure, and 50-70 mg/kg ketamine hydrochloride were administered intramuscularly. A midline laparotomy incision was performed after the purification of the abdominal wall with 10% povidone-iodine solution. As described previously,[5] atraumatic microvascular clamp was placed across the superior mesenteric artery just after its origin from the aorta. Mesenteric ischemia was confirmed when the mesenteric pulsations were lost, and the intestinal segment became pale. The bowel was returned to the abdominal cavity, and the incision was closed with interrupted 4-0 silk sutures. After 45 minutes ischemia, a relaparotomy was performed, and the microvascular clamp on the artery was removed for 60 minutes reperfusion. Mesenteric reperfusion was confirmed with the return of pulsation and color. The bowel was then returned to the abdominal cavity once more, and the incision was closed with interrupted 4-0 silk suture. After 60 minutes (reperfusion time), stitches were opened and all of the rats' intracardiac blood samples were taken into the tubes with heparin for biochemical studies. The blood samples were kept in a temperature of -80°C, until the subsequent procedure. Five cm of the proximal ileal segment were resected, washed with 0.9% saline solution, and placed in 10% formaldehyde for histopathologic analysis. Another 5 cm of the remaining proximal ileal segment was resected, washed with 0.9% saline, dried with blotter, covered with an aluminum leaf, and kept in a temperature of -80°C, until the subsequent procedure. The animals were sacrificed with cervical dislocation. The tissue specimens were fixed with 10% formaldehyde, then dehydrated, and embedded in paraffin wax. The samples of intestine were sectioned and stained with hematoxylin eosin (H&E), and submitted for histopathologic evaluation by a histopathologist in a blinded fashion using a light microscope. Histopathologic examination was performed using the scoring system of Chiu et al,[6] described briefly as follows: grade 0 - normal mucosal villi; grade one - development of a subepithelial space, usually at the tip of the villus, with capillary congestion; grade 2 - extension of the subepithelial space with moderate lifting of the epithelial layer; grade 3 - massive epithelial lifting down the sides of the villi; grade 4 - denuded villi with the lamina propria dilated capillaries exposed, increased cellularity of the lamina propria; grade 5 - digestion and disintegration of the lamina propria, hemorrhage, and ulceration. The levels of GSH were evaluated both in the washed erythrocytes and intestinal tissue samples. Besides, malondialdehyde (MDA), superoxide dismutase (SOD), and nitric oxide

(NO) levels were evaluated in the plasma samples and intestinal tissue samples. The spectrophotometric method previously described by Buege and Aust,[7] were used to asses the levels of MDA. This method was based on the formation of a red color as a result of the reaction between MDA and thiobarbituric acid, one of the products of lipid peroxidation in a hot medium. The levels of MDA are denominated as nmol/mL and nmol/protein. The detection limits of MDA assay are 0.90 nmol/mL and 0.10 nmol/ protein. In the tissue and plasma samples, SOD activity was evaluated by the method generated by Sun et al[8] based on the inhibition of the nitroblue tetrazolium (NBT) reduction of super oxide anions by SOD, which is formed by the system of xanthine oxidase.[8] A unit of SOD points out the enzyme activity inhibiting 50% of the NBT reduction by 50%. The activity of SOD is denominated as U/mL, and U/ mg protein. The detection limits of the SOD assay are 25.0 U/mL, and 0.70 U/mg protein. In the erythrocyte and tissue samples, GSH levels were measured by the method of Beutler et al[9] based on the formation of reduced yellow color chromogen, which is absorbed in 412 nm in an enclosed medium containing erythrocyte hemosylate deproteinized with salt sedimentation with the compound of 5, 5'-(Dithiobis)-2-nitrobenzoic acid-GSH.[9] The level of GSH is denominated as µmol/g hemoglobin (Hb) and mg/mg protein. The detection limits of GSH assay are 2.20 µmol/g Hb, and 7.30 mg/mg protein. The levels of nitrite and nitrate were analyzed by a photometric endpoint determination. Nitrate was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme, nitrate reductase. The nitrite reacted with sulphanilamide and N-(1-naphtyl)-ethylene-diamine dihydrochloride to give a red-violet diazo dye. The diazo dye was measured by its absorbance in the visible range at 540 nm.[10] The levels of NO are denominated as µmol/L, and µmol/g wet tissue. The detection limits of NO assay are 18.24 µmol/L, and 330 µmol/g wet tissue.

All data were presented in mean±SD. The Kolmogorov- Smirnov test was used to assess the normality of the continuous data. All data were analyzed and accepted to be normal. For all variables, the statistical differences between the groups were tested using one-way analysis of variance. Multiple comparisons were made using the Bonferroni post-hoc test. The data with the values of $p < 0.05$ were considered as statistically significant. Statistical package for Social Sciences/PC+ version 11.0 (SPSS Inc., Chicago, Illinois) was used for the statistical analysis.

**Results.** The mean histopathologic injury score in the I/R group was significantly higher than in the control and ischemia groups ($p < 0.001$). In the NAC-treated groups, the histopathologic scores were significantly lower than in all I/R groups ($p < 0.001$). The histopathologic findings are shown in **Figures 1, 2a, and 2b**. When the IR group was compared to the control group, the tissue GSH level ($p < 0.001$), and SOD ($p < 0.01$) activity were significantly lower, and MDA and NO levels were higher ($p < 0.001$). The NAC treatment significantly lowered MDA ($p < 0.001$) and NO ($p < 0.001$) levels which have been induced by IR. The NAC treatment led to significant increases in the mean tissue GSH level ($p < 0.001$), however, the increase in SOD activity was not significant. The values of the tissue GSH, MDA, NO levels, and SOD activities are shown in **Table 1**. When the IR group was compared to the control group, plasma GSH levels were significantly lower ($p < 0.001$), while SOD activity ($p < 0.001$), MDA ($p < 0.001$) and NO levels ($p < 0.01$) were higher. The NAC treatment significantly increased the MDA, and the GSH was reduced by the injury itself ($p < 0.001$). However, no significant effect of NAC treatment was found on the plasma SOD activity and NO levels. The values of plasma MDA, NO levels, and SOD activities and erythrocyte GSH levels are shown in **Table 2**.
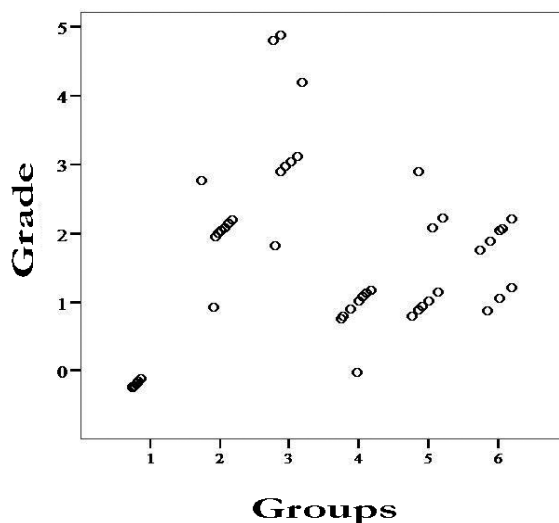


**Figure 1 -** The histopathological injury scores. Group 1 - control, Group 2 - ischemia (ischemia for 45 minutes), Group 3 - ischemia-reperfusion (ischemia for 45 minutes and reperfusion for 60 minutes), Group 4 - ischemia + N-acetylcysteine (ischemia for 45 minutes), Group 5 - ischemia-reperfusion + N-acetylcysteine (ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered in the beginning of ischemia), Group 6 - reperfusion + N-acetylcysteine (ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered in the start of reperfusion) (n=8 for each group).
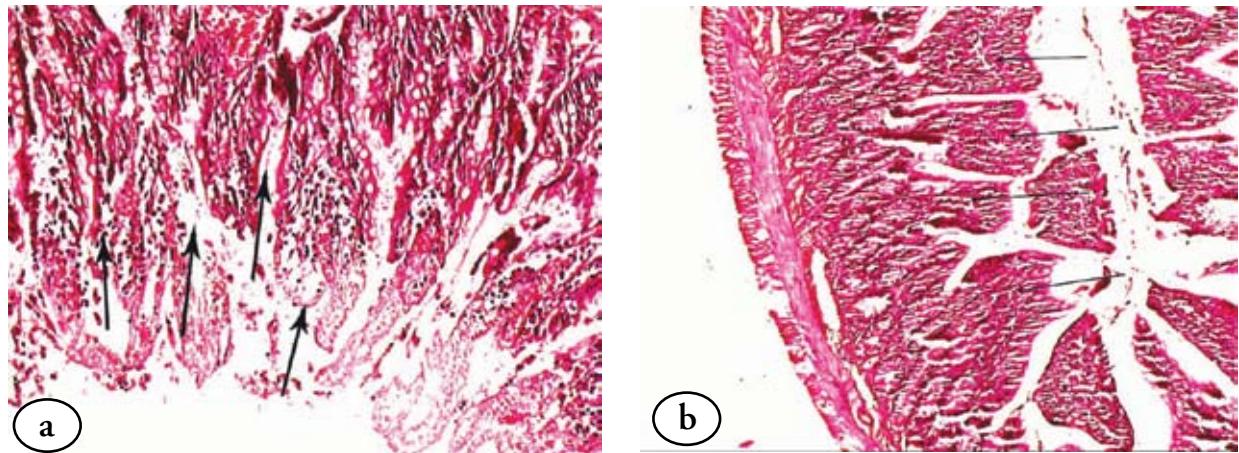
**Figure 2 -** The histopathological findings showing **a)** the injury (arrows) occurring in IR group, capillary dilation and an increase in the permeability of lamina propria and a superficial necrosis and peeling of villus are observed (HE, x100) and the **b)** decrease of the injury (arrows) in IRN group. Capillary dilation in lamina propria with the minimal edema expansion in subepithelial area are observed (HE, X50). IRN - Ischemia+N-acetylcysteine group, IR - Ischemia-Reperfusion group, H&E - hematoxylin eosin

**Table 1 -** The values of tissue MDA (nmol/protein), GSH (mg/mg protein) NO levels (nmol/g wet tissue) and SOD (U/mg protein) activity for each group.

| Tissue | Control n=8 | Ischemia[a] n=8 | Ischemia reperfusion[b] n=8 | Ischemia+ N-acetylcysteine[c] n=8 | Ischemia-reperfusion+ N-acetylcysteine[d] n=8 | Reperfusion+ N-acetylcysteine[e] n=8 |
|---|---|---|---|---|---|---|
| GSH | 23.48±3.43 | 17.82±4.14 | 10.97±3.50[†††] | 17.98±4.20** | 15.90±3.93* | 22.12±4.10*** [§] |
| SOD | 1.17±0.21 | 0.95±0.11 | 0.87±0.17* | 1.19±0.17** | 1.07±0.11 | 1.07±0.19 |
| MDA | 0.25±0.08 | 0.46±0.09[‡] | 0.57±0.11[†††] | 0.32±0.08 | 0.40±0.08** | 0.29±0.07*** |
| NO | 380.37±24.58 | 511.75±70.84 | 717.75±42.48[†††] | 483.25±45.88*** | 604.75±58.37** | 528.88±60.71*** |

Values are expressed as mean ± SD, *$p<0.05$ compared with C, **$p<0.01$ compared with IR, ***$p<0.001$ compared with IR, [†††]$p<0.001$ compared with C, [‡]$p<0.05$ compared with IN, [§]$p<0.05$ compared with IRN group. [a]ischemia for 45 minutes, [b]ischemia for 45 minutes and reperfusion for 60 minutes, [c]ischemia for 45 minutes, [d]ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered the beginning of ischemia), [e]ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered the start of reperfusion. MDA - Malondialdehyde SOD - superoxide dismutase, GSH - glutathione, NO - nitric oxide, NAC - N-acetylcysteine

**Table 2 -** The values of plasma MDA (nmol/mL), NO levels (µmol/L) and SOD (U/mL) activity in plasma and erythrocyte GSH (µmol/gr.Hb) for each group.

| Plasma | Control n=8 | Ischemia[a] n=8 | Ischemia reperfusion[b] n=8 | Ischemia+ N-acetylcysteine[c] n=8 | Ischemia-reperfusion+ N-acetylcysteine[d] n=8 | Reperfusion+ N-acetylcysteine[e] n=8 |
|---|---|---|---|---|---|---|
| GSH | 4.34±0.36 | 3.23±0.33[†††] | 2.85±0.29[†††] | 3.75±0.37*** | 3.49±0.38 ** | 3.98±0.35*** |
| SOD | 30.15±3.15 | 30.74±2.69 | 35.99±2.69[‡‡] | 34.06±3.01 | 34.54±2.81 | 33.02±3.70 |
| MDA | 1.46±0.35 | 5.11±0.52* [†††] | 5.34±0.77[†††] | 4.07±0.46*** | 3.84±0.59*** | 1.78±0.48*** [§§§] |
| NO | 23.02±2.96 | 47.15±4.73[†††] | 44.73±3.9[†††] | 40.41±5.40[†††] | 39.26±5.51[†††] | 41.79±4.08[†††] |

Values are expressed as mean ± SD, *$p<0.05$ compared with IN, **$p<0.01$ compared with IR, ***$p<0.001$ compared with IR, [‡‡]$p<0.01$ compared with C, [†††]$p<0.001$ compared with C, [§§§]$p<0.001$ compared with IRN group. [a]ischemia for 45 minutes, [b]ischemia for 45 minutes and reperfusion for 60 minutes, [c]ischemia for 45 minutes, [d]ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered the beginning of ischemia, [e]ischemia for 45 minutes and reperfusion for 60 minutes and NAC was administered the start of reperfusion. MDA - Malondialdehyde, SOD - superoxide dismutase, GSH - glutathione, NO - nitric oxide, NAC - N-acetylcysteine

**Discussion.** In different organs including the intestines, metabolic and functional problems may occur, secondary to damage induced by ischemia, and also by reperfusion. Histological assessment using a microscopic scoring system has been accepted as a good standard in the evaluation of I/R injury in the intestinal tissue.[10,11] In the present study, the most severe injury was observed in the reperfusion group and the histopathological changes were discrete, when compared to the ischemic group. This may be considered as an indicator of the successfully created I/R injury in the small intestines in our experimental model. Oxidative stress plays an important role in the intestinal I/R injury. In the intestinal tissue exposed to I/R, activated neutrophils induce tissue injury through the production and release of reactive oxygen derivatives and cytotoxic proteins into the extracellular fluid, initiating the inflammatory cascades that trigger the radical induced I/R injury.[12,13] The synthesis of the reactive oxygen species, neutrophil infiltration, and the release of inflammatory reaction mediators during the posterior reperfusion, exacerbate ischemia-induced mucosal injury.[14] In our study, I/R injury induced the increase of the tissue MDA and NO levels, and the decrease of the tissue GSH levels, and the SOD activity when compared to the controls (**Table 1**). Ischemia/reperfusion injury significantly induced the increase of the plasma MDA, NO, and SOD activities, and the decrease of the erythrocyte GSH levels when compared to the controls (**Table 2**). These results suggest that increased oxidative stress in intestinal I/R may be responsible for the I/R injury and endogenous antioxidant enzymes, such as SOD, and GSH may not be sufficient to prevent the intestinal I/R injury. N-acetylcysteine is a thiol-containing compound that detoxifies the free radicals by non-enzymatic reactions, and is deacetylated to form cysteine, which supports the biosynthesis of GSH, one of the most important components of the intracellular antioxidant systems.[15] In the literature, studies on the preventive effect of NAC against intestinal I/R injury are limited.[16-18] In this study, NAC administration improved the I/R histopathologic damages in the intestinal tissue, decreased the tissue MDA and NO levels, and increased the tissue GSH levels. Similarly, NAC treatment decreased the elevated plasma MDA levels and increased the diminished erythrocyte GSH levels. However, no statistically significant difference was observed in the tissue SOD activity (**Table 1**), and in plasma SOD activity and NO levels with the NAC treatment (**Table 2**). In the literature, the effects of NAC on pathological score,[17,18] antioxidant enzymes levels or activity[16] in I/R intestine have been reported. Nevertheless, there is no report suggesting on the dosage, and the timing of NAC administration. *In vitro* and *in vivo* studies have shown that NAC acts as a cysteine prodrug, and a GSH precursor.[19] In our study, tissue and plasma GSH levels were used to show the efficiency of NAC therapy in intestinal I/R in rats. The administration time of NAC also effected the protective effects on intestinal I/R in rats. Among the IR groups, the group with GSH level closest to the normal values received the treatment at the beginning of the reperfusion. Therefore, this result suggests that the application of NAC with reperfusion may be more effective than the application with ischemia in intestinal I/R.

The indicator of oxidative stress in the cell is the levels of the marks of the lipid peroxidation and the final product is MDA.[20,21] Malondialdehyde can be titered in both tissue and blood, and its concentration is directly proportional to the cell damage caused by free radicals.[22] The MDA levels are considered to be a reliable indicator of a certain I/R event, increasing with the severity of the injury.[22,23] In this study, the highest value of MDA levels were found in the IR group, which suggested that the most serious damage occurred in the IR group. Additionally, tissue and plasma MDA levels were low in rats treated with NAC. However, when plasma MDA levels according to the application time were considered, giving NAC at the beginning of reperfusion, was found to be more decreasing than giving it at the beginning of ischemia. These results demonstrated that NAC treatment reduced the damage in the ileum injured by I/R. As a result, the antioxidant effects of NAC are well documented by the *in vivo* and *in vitro* studies. It successfully inhibits the oxidative stress at both high and low concentrations, under acute (*in vitro*), and chronic administration (*in vivo*).[15] In a study by Börjesson et al,[24] the beneficial effects of NAC was attributed to its ability to reduce the reactive oxygen species, rather than replenishing intracellular GSH stores, since the time of administration was after the onset of reperfusion, and the production of reactive oxygen species has already been manifested. In our study, the administration of NAC improved the replenishing of the intracellular GSH stores, which has been reduced by the I/R injury. This improvement was also supported by the decrease of MDA levels, which indicate an ischemia-reperfusion event.

In conclusion, NAC administration causes an improvement in the histopathologic findings of I/R damages. The N-acetylcysteine treatment protects the antioxidant enzymes in the tissue, plasma, and the erythrocytes, which are crucially important in the intestinal I/R injury in rats. It was observed that administration of NAC at the beginning of reperfusion had positive effects on increasing the tissue GSH and plasma MDA levels. Further clinical and experimental investigations are required, to determine the potential

effects of NAC on intestinal I/R injury at different doses, at different administration times, and treatment durations.▪

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