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Effects of N-acetylcysteine on liver remote injury after skeletal muscle ischemia reperfusion in rats

Mohammad Ashrafzadeh Takhtfooladi[1], Gholamreza Jahanshahi[2], Amirali Jahanshahi[1], Amir Sotoudeh[3], Omidreza Samiee Amlashi[1], Amin Allahverdi[1]

[1]Department of Surgery, Islamic Azad University Faculty of Specialized Veterinary Science, Science and Research Branch, Tehran, Islamic Republic of Iran
[2]Department of Oral & Maxillofacial Pathology, Isfahan University of Medical Science Faculty of Dentistry, Isfahan, Islamic Republic of Iran
[3]Kahnooj Branch, Islamic Azad University Faculty of Veterinary Science, Kerman, Islamic Republic of Iran

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ABSTRACT

Background/Aims: This study evaluated the effects of N-acetylcysteine as a scavenger of radical oxygen species on liver injury as a remote organ after skeletal muscle ischemia reperfusion.

Materials and Methods: Twenty male Wistar rats were allocated randomly into two experimental groups: ischemia reperfusion (I/R) and ischemia reperfusion + N-acetylcysteine (I/R+NAC). All animals were undergone 2h of ischemia by occlusion femoral artery and 24h of reperfusion. Rats that were treated with N-acetylcysteine given intravenously at a dose of 150 mg/kg, immediately before reperfusion. Serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured. Livers were harvested for histopathological and biochemical studies. Liver tissue malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) activity were assayed.

Results: The ALT and AST values were significantly lower in I/R+NAC group. Hepatic MDA level and MPO activity were significantly increased in I/R group. The levels of GSH in liver tissue were significantly depressed by ischemia reperfusion. Liver histopathologic study in I/R group showed enlarged sinusoids, sinusoidal congestion, cytoplasmic vacuolation, cellular degenerative changes and necrosis. Histopathologically, there was a significant difference between two groups.

Conclusion: Histopatological and biochemical results have shown that N-acetylcysteine was able to protect liver from skeletal muscle ischemia reperfusion injury.

Keywords: N-acetylcysteine, skeletal muscle, ischemia reperfusion, liver remote injury

INTRODUCTION

Ischemia reperfusion injury describes the clinically prevalent finding that tissue ischemia with inadequate oxygen supply followed by reperfusion initiates a wide and complex array of inflammatory responses that may cause damage to the affected tissues or remote organs (1). A devastating consequence of tissue reperfusion is the development of damage in organs uninvolved in the initial ischemic insult. Multiple organ dysfunction syndrome, is the leading cause of death in patients and is a documented consequence of liver (2,3), gut (4,5), skeletal muscle (6) and aortic occlusion reperfusion (7,8) and circulatory shock. Conditions under which transient ischemia damage is encountered include the different forms of acute vascular occlusions with the respective reperfusion strategies but also routine surgical procedures and major trauma or shock (1).

Several mechanisms have been offered to describe the ischemia reperfusion injuries in affected tissues and remote organs; however, most attention has focused on the role of oxidants and leukocyte activation (9,10).

N-acetylcysteine is a small molecule that made from the amino acid cysteine joined to an acetyl group. N-acetylcysteine is a source of sulfhydryl groups and is

Address for Correspondence: Ashrafzadeh Takhtfooladi Mohammad, Department of Surgery, Islamic Azad University Faculty of Specialized Veterinary Science, Science and Research Branch, Tehran, Islamic Republic of Iran
E-mail: dr_ashrafzadeh@yahoo.com
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converted in the body into metabolites capable of stimulating glutathione synthesis, promoting detoxification, and acting directly as a free radical scavenger (11-13). Based on these features, N-acetylcysteine is widely used in clinical practice as an antioxidant.

In previous studies, administered N-acetylcysteine was shown to protect against ischemia reperfusion injuries in affected tissues and remote organ after skeletal muscle ischemia reperfusion and it was suggested that this protective effect may be due to its ability to scavenge free radicals (14-17). However, the protective effect of N-acetylcysteine on liver from skeletal muscle ischemia reperfusion injury was not studied to date.

In this present study, the protective effect of N-acetylcysteine on liver as a remote organ damage after transient skeletal muscle ischemia was examined by assessing functional, histological and biochemical changes in rat model.

MATERIALS AND METHODS

All animal experiments were conducted according to the guidelines provided by the animal committee of the Azad University and was carried out in accordance with guidelines for use of laboratory animals.

Twenty male Wistar rats weighing 250-300 g were used in this study. All rats were kept at a constant room temperature under standard conditions with food and water ad libitum. Animals were allocated randomly into two experimental groups: group ischemia reperfusion (I/R) and group ischemia reperfusion plus N-acetylcysteine (I/R+NAC).

Anesthesia was induced using intramuscular ketamine (50 mg/kg) plus xylazine (10 mg/kg). After induction of anesthesia, the left hind limb was prepared for sterile surgery. A skin incision was made on medial surface of the left hind limb. Femoral artery was isolated from the surrounding structures and was clamped with a mini bulldog forceps for 2 hours.

Before clamping of the femoral artery, 250 IU heparin was administered via the jugular vein to prevent clotting.

Rats were maintained in dorsal recumbence and kept anesthetized with additional doses injection (if necessary) throughout the duration of the ischemic period. Body temperature was maintained with a heating pad under anesthesia. In I/R+NAC group, N-acetylcysteine (150 mg/kg) was injected intravenously immediately before reperfusion. Following the ischemic period, the vascular forceps was removed and then surgical site was routinely closed with nylon 3/0 threads in two layers. Fluid losses were replaced by intraperitonealy administration of 5 ml of warm (37˚C) isotonic saline. Rats were returned to their cages with food and water ad libitum during the reperfusion period. The analgesic nalbuphine hydrochloride (2 mg/kg SC) was used during observation time.

After 24h of reperfusion, the blood samples were collected from jugular vein and submitted for evaluation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values to assess liver functions. The serum levels of AST and ALT were measured with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China). Rats were euthanized with an overdose of pentobarbital injection (300 mg/kg IP) and liver harvested rapidly for histopathological and biochemical analysis. Small pieces of liver tissue were placed in 10% formalin solution and processed routinely by embedding in paraffin then tissues were sectioned in 5 µm pieces and stained with Hematoxylin-Eosin stain.

Grading of severity of liver injury was carried out by a pathologist who was blinded to the experiment and data. Hepatic injury was graded into four grades as follows: grade 0, no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (18). A total of five slides from each liver sample were randomly screened and the mean was accepted as the representative value of the sample.

The remaining hepatic tissue samples were stored at -70 ˚C. Afterwards, tissue malondialdehyde (MDA) levels, an end product of lipid peroxidation, glutathione (GSH), a key antioxidant, and tissue-associated myeloperoxidase (MPO) activity, as indirect evidence of neutrophil infiltration, were measured in these samples.

The MDA levels were assayed for products of lipid peroxidation as described by Sener et al (19) and expressed as nmol MDA/g tissue. GSH was analyzed spectrophotometrically which was based on the use of Ellman's reagent (20). Results were expressed µmol GSH/g tissue. MPO activity was measured in liver tissue in a procedure similar to that documented by Hillegas et al (21). MPO activity was expressed as U/g tissue.

The Mann-Whitney U-test and T-test were employed to analyze two groups consecutively. Values of $P < 0.05$ were considered as statistically significant. Statistical analyses were carried out using SPSS statistical software (version 16.0).

RESULTS

All rats well tolerated operation and survived until the final study period. Data belonging to AST and ALT measurements from blood samples are shown in Table 1. AST and ALT levels were significantly higher ($p<0.05$) in the I/R group when compared with the I/R+NAC group.

The histopathological evaluations of the liver sections from two groups are shown in Table II. In the liver sections of I/R group cellular degenerative changes were intense, sinusoidal congestion, enlarged sinusoids, cytoplasmic vacuolation and

necrosis were observed (Figure 1). Group I/R+NAC represent well preserved liver hepatocytes and sinusoids. No congestion is noticed in sinusoids (Figure 2). Histopathologic examination confirmed the extent of liver changes in the I/R+NAC group were significantly lower ($p<0.05$) than I/R group.

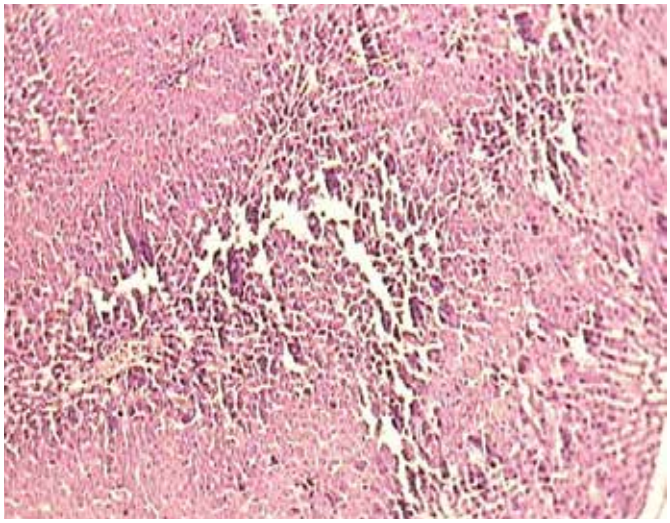

Figure 1. Light microscopic view of liver tissues from IR group showing loss of arrangement and necrosis (magnification of 10×10, H&E staining).

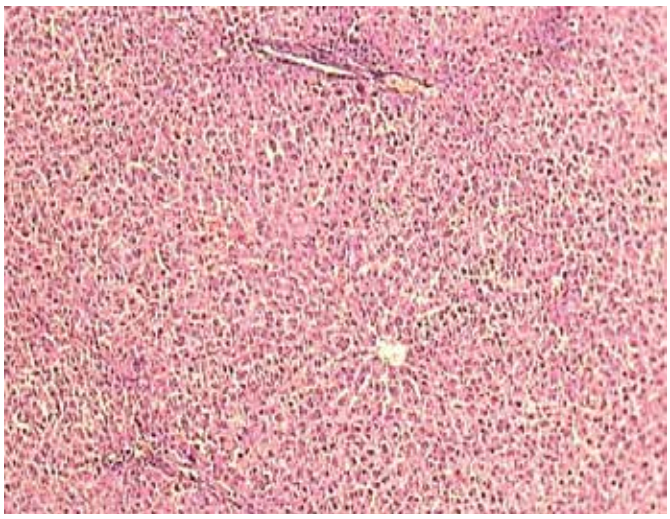

Figure 2. Light microscopic view of liver tissues from IR+NAC group showing well-preserved liver parenchyme (magnification of 10×10, H&E staining).

Table 1. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations (units/Liter)

Group	n	AST (Mean±SD*)	ALT (Mean±SD*)
I	10	354.9±11.9	98±3.3
II	10	189.5±5.8	52±2.55

* There is significant difference (p=.000) between two groups.

Table 2. Scores of liver histological changes

Group	N	Mean±SD*
I	10	2.4±0.51
II	10	0.6±0.51

* There is significant difference (p=.000) between two groups.

Hepatic MDA level and MPO activity were significantly increased in I/R group (Figures 3 and 4). The levels of GSH in liver tissue were significantly depressed by ischemia reperfusion (Figure 5).

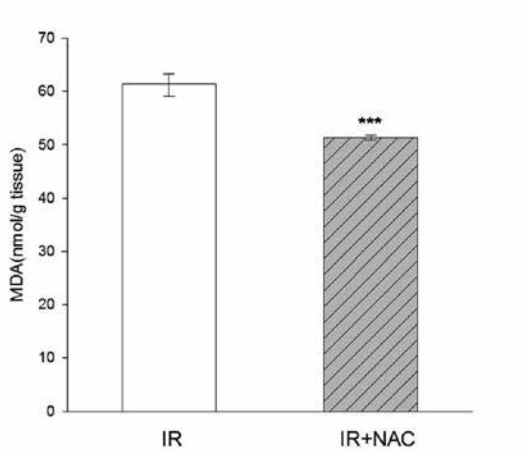

Figure 3. The malondialdehyde (MDA) levels of liver tissues. Extent of lipid peroxidation estimated as MDA. p=.000.

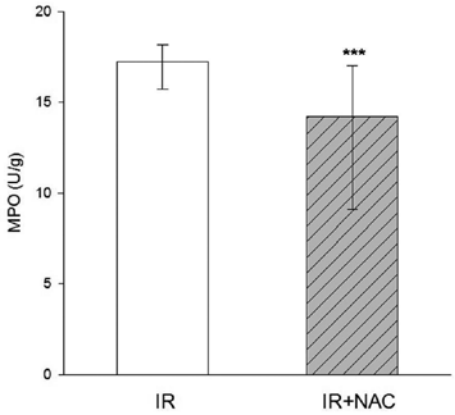

Figure 4. The myeloperoxidase (MPO) activity in hepatic tissues. p=.000.

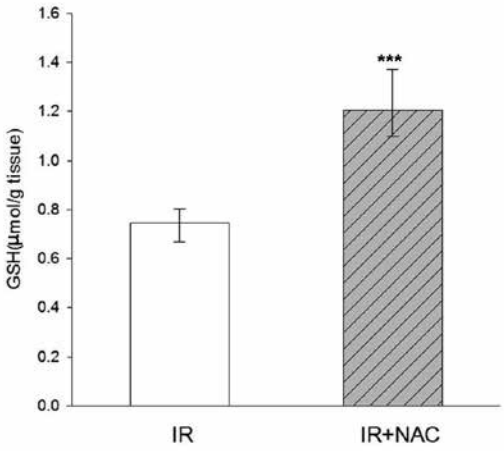

Figure 5. The glutathione (GSH) levels of liver tissues. p=.000.

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DISCUSSION

Ischemia reperfusion injury is a critical and fatal problem at the case of obstruction diseases. Ischemia reperfusion injuries not only affect local tissue but also result in life-threatening damage to remote organs.

The various orthopedic conditions in which extremity ischemia can occur include arterial clamping during surgery, use of a tourniquet during a limb operation and constriction of a limb under wreckage in a disaster. For recovery, blood reperfusion of the limb is inevitably required. Many researchers have investigated the mechanisms and treatments of ischemia reperfusion injury (22). Previous studies suggest that reperfusion following ischemia stimulates production of reactive oxygen species and triggers the participation of neutrophils causing an inflammatory reaction (22,23). In a normal situation, superoxide dismutase, which converts superoxide into hydrogen peroxide, acts to prevent superoxide from damaging tissue (23), but in reperfusion injury, these natural defenses may be overcome and hydrogen peroxide is converted into the hydroxyl radical, which can damage to the biological molecules including amino acids, nucleic acids and membrane transport proteins (24). The most important damage caused by oxygen free radicals is owing to lipid peroxidation, which results in cell structural and functional alterations (25).

Several methods have been used to define the role of netrophils in reperfusion tissue injury. MPO plays a fundamental role in oxidant production by netrophils. Neutrophils are a potential source of oxygen free radicals (26). Grisham et al. (27) have examined the influence of ischemia reperfusion on neutrophil fluxes in cat intestinal mucosa using tissue-associated MPO activity. In this study N-acetylcysteine inhibited MPO activity which was increased by ischemia reperfusion.

Glutathione provides major protection in oxidative injury by participating in the cellular defense systems against oxidative damage (28). GSH scavenges O_2^- and protects protein thiol groups from oxidation. The present study showed depletion of liver tissue GSH which was restored by N-acetylcysteine treatment.

In this study skeletal muscle ischemia reperfusion caused significant increases in liver tissue MDA which is products of lipid peroxidation. N-acetylcysteine treatment abolished the increase in MDA, probably in part by scavenging the very reactive hydroxyl radicals.

Experimental animal studies have demonstrated the efficacy of antioxidant therapy in attenuating or preventing ischemia reperfusion injury, including the use melatonin, vitamin E, superoxide dismutase, catalase, mannitol, allopurinol, iron chelating compounds, angiotensin- converting enzyme inhibitors, calcium channel antagonists or N-acetylcysteine (14,15,29).

N-acetylcysteine is a simple water soluble molecule that contains a sulfhydryl residue. It has been approved for the prevention of hepatic damage following acetaminophen poisoning (30). This documented effect has been attributed to the restoration of intracellular glutathione levels (31,32), required for detoxification of toxic metabolites of acetaminophen-derived. N-acetylcysteine also may attenuate the course of hepatorenal syndrome, a renal vasoconstrictive response of indeterminate nature that develops during advanced liver failure. This effect, shown in experimental settings (33) and in a preliminary clinical report (34), could imply a better preservation of liver function.

In this study liver was examined to assess remote organ injury after skeletal muscle ischemia reperfusion. Both ALT and AST, which have been used as markers of liver pathology (35), are also found in skeletal muscle and thus increases in their values may be attributed to levels in skeletal muscle, rather than to liver injury alone (36). The liver histological changes suggested that increases in these enzymes reflect hepatic injury. Sagara et al. (37) showed that liver function deteriorated due to limb ischemia reperfusion and that the extent of deterioration corresponded to the time period of ischemia.

In an experimental study Nagasaki et al. (38) showed that N-acetylcysteine prevented hepatic injury after liver ischemia reperfusion. Sener et al related administration of melatonin and N-acetylcysteine prevented hepatic malfunction and inhibited the oxidative stress and accumulation of neutrophils in the damaged hepatic tissue, these agents appear to play a cytoprotective role in liver insulted by ischemia reperfusion (19). In this study, our data demonstrate that N-acetylcysteine significantly decreases the severity of liver injury after skeletal muscle ischemia reperfusion in rats.

In conclusion, this study confirmed that transient ischemia in skeletal muscle leaded to liver functional and histological changes in I/R group. However, administration of the N-acetylcysteine treatment significantly decreased hepatic injury induced by skeletal muscle ischemia reperfusion according to our histological and biological findings. These results suggest the possibility of clinical application of N-acetylcysteine on hepatic injury induced by ischemia reperfusion. In future studies, different dosages and alternate time protocols of N-acetylcysteine administration should be investigated.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Dorweiler B, Pruefer D, Andrasi TB, Maksan SM, Schmiedt W, Neufang A, Vahl CF. Ischemia-reperfusion injury pathophysiology and clinical implications. Eur J Trauma Emerg Surg 2007; 6: 600-12.
2. Liu DL, Jeppsson B, Hakansson CH, Odselius R. Multiplesystem organ damage resulting from prolonged hepatic inflow interruption. Arch Surg 1996; 131: 442-7.
3. Weinbroum AA, Nielson VG, Tan S, Gelman S, Matalon S, Skinner KA, Bradley E Jr, Parks DA. Liver ischemia-reperfusion increases

pulmonary permeability in rat: role of circulating xanthine-oxidase. Am J Physiol 1995; 268: 988-96.
4. Carden DL, Young JA, Granger DN. Pulmonary microvascular injury after intestinal ischemia-reperfusion: role of P-selectin. J Appl Physiol 1993; 75: 2529-34.
5. Terada LS, Dormish JJ, Shanley PF, Leff JA, Anderson BO, Repine JE. Circulation xanthine oxidase mediates lung neutrophil sequestration after intestinal ischemia-reperfusion. Am J Physiol 1992; 263: 394- 401.
6. Fantini GA, Conte MS. Pulmonary failure following lower torsoischemia: clinical evidence for a remote effect of reperfusion injury. Am Surg 1995; 61: 316-9.
7. Barry MC, Wang JH, Kelly CJ, Sheehan SJ, Redmond HP, Bouchier-Hayes DJ. Plasma factors augment neutrophil and endothelial activation during aortic surgery. Eur J Vasc Endovasc Surg 1997; 13: 381-7.
8. Harward TR, Welborn MB, Martin TD, Flynn TC, Huber TS, Moldawer LL, Seeger JM. Visceral ischemia and organ dysfunction after thoracoabdominal aortic aneurysm repair: a clinical and cost analysis. Ann Surg 1996; 223: 729-34.
9. Granger DN. Role of xanthine oxidase and granulocytes in ischemia±reperfusion injury. Am J Physiol 1988; 255: 1269 -75.
10. Granger DN, Korthuis RJ. Physiologic mechanisms of postischemic tissue injury. Annu Rev Physiol 1995; 57: 311-32.
11. De Vries N, De Flora S. N-acetyl-l-cysteine. J Cell Biochem. 1993; 17: 270-7.
12. Holodines MR. Clinical pharmacokinetics of N-acetylcysteine. Clin pharmacokinetics. 1991; 20: 123-34.
13. Cuzzocrea S, Mazzon E, Costantino G, Serraino I, De Sarro A, Caputi AP. Effedts of N-acetylcysteine in a rat model of ischemia and reperfusion injury. Cardiovasc Res 2000; 47: 537-48.
14. Takhtfooladi MA, Jahanshahi A, Sotoudeh A, et al. Synergistic protective effect of ischemic preconditioning technique in combination of N-acetylcysteine on skeletal muscle ischemia reperfusion injury in rat; Histopathological study. Eur J Exp Biol 2012; 2: 1766-70.
15. Takhtfooladi MA, Jahanshahi A, Sotoudeh A, Jahanshahi GH, Aslani K, Khansari M, Adibi S, Takhtfooladi HA. Effects of N-acetylcysteine upon the rat serum enzyme changes in skeletal muscle ischemia reperfusion. Annals of Biological Research 2012; 3: 4099-102.
16. Takhtfooladi MA, Jahanshahi A, Jahanshahi GH, Sotoudeh A, Takhtfooladi HA, Khansari M. Protective effect of N-acetylcysteine on kidney as a remote organ after skeletal muscle ischemia-reperfusion. Acta Cir Bras 2012; 27: 611-5.
17. Sotoudeh A, Takhtfooladi MA, Jahanshahi A, Khiabanian Asl AH, Takhtfooladi HA, Khansari M. Effect of N-acetylcysteine on lung injury induced by skeletal muscle ischemia-reperfusion: histopathological study in rat model. Acta Cir Bras 2012; 27: 168-71.
18. Camargo CAJ, Madden JF, Gao W, Selvan RS, Clavien PA. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. Hepatology 1997; 26: 1513-20.
19. Sener G, Tosun O, Şehirli Ö, Kaçmaz A, Arbak S, Ersoy Y, Ayanoğlu-Dülger G. Melatonin and N-acetylcysteine have beneficial effects during hepatic ischemia and reperfusion. Life Sci 2003; 72: 2707-18.
20. Beutler E. Glutathione in red blood cell metabolism. A manuel of biochemical methods. New York: Grune and Stratton; 1975. p.112-4.
21. Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990; 24: 285-95.
22. Grace PA. Ischaemia-reperfusion injury. Br J Surg 1994; 81: 637-47.
23. Anaya-Prado R, Toledo-Pereyra LH, Lentsch AB, Ward PA. Ischemia/reperfusion injury. J Surg Res 2002; 105: 248-58.
24. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 1992; 119: 598-620.
25. Slater TF. Free-radical mechanisms in tissue injury. Biochem J 1984; 222: 1-15.
26. Weiss SJ, Ward PA. Immune complex induced generation of oxygen metabolites by human neutrophils. J Immunol 1982; 129: 309-13.
27. Grisham MB, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am J Physiol 1986; 251(4 Pt 1): G567-74.
28. Ross D. Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther 1988; 37: 231-49.
29. Maxwell SR, Lip GY. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. Int J Cardiol 1997; 58: 95-117.
30. Smilkstein MJ, Knapp GL, Kulig KW, Rumack BH. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976 to 1985). N Engl J Med 1988; 319: 1557-62.
31. Corcoran GB, Wong BK. Role of glutathione in prevention of acetaminophen-induced hepatotoxicity by N-acetyl-L-cysteine in vivo: studies with N-acetyl-D-cysteine in mice. J Pharmacol Exp Ther 1986; 238: 54-61.
32. Grattagliano I, Vendemiale G, Deiss VB, Junker E, Lauterburg BH. Effect of oral and intravenous S,N-diacetylcysteine monoethyl ester on circulating and hepatic sulfhydryls in the rat. J Pharmacol Exp Ther 2000; 294: 155-9.
33. Simko V, Michael S, Katz J, Oberstein E, Popescu A. Protective effect of oral acetyl- cysteine against the hepatorenal toxicity of carbon tetrachloride potentiated by ethyl alcohol. Alcohol Clin Exp Res. 1992; 16: 795-9.
34. Holt S, Goodier D, Marley R, Patch D, Burroughs A, Fernando B, Harry D, Moore K: Improvement in renal function in hepatorenal syndrome with N-acetylcysteine. Lancet 1999; 353: 294-5.
35. Holoman J, Glasa J, Galbavy S, et al. Serum markers of liver fibrogenesis, and liver histology findings in patients with chronic liver diseases. Bratisl Lek Listy 2002; 103: 70-5.
36. Tay SK, Ong HT, Low PS. Transaminitis in Duchenne's muscular dystrophy. Ann Acad Med Singapore 2000; 29: 719-22.
37. Sagar G, Ogikubo O, Takeyama, Tada T, Suzuki N, Okamoto H, Otsuka T. Comparison of effects of ischemia period in rat limb ischemia/reperfusion injury. Nagoya Med J 2009; 50: 83-91.
38. Nagasaki H, Nakano H, Boudjema K, Jaeck D, Alexandre E, Baek Y, Kitamura N, Yamaguchi M, Kumada K. Efficacy of preconditioning with N-Acetylcysteine against reperfusion injury after prolonged cold ischemia in rat liver in which glutathione had been reduced by buthionine suphoximine. Eur J Surg 1998; 164: 139-46.