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Combination Therapy of N-Acetylcysteine and Simvastatin Reduce Malondialdehyde(MDA) Levels in Animal Models with Ischemic/Reperfusion Injury (IRI)

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ABSTRACT

Ischemic/reperfusion injury (IRI), refers to the damage caused by the restoration of blood supply to previously ischemic tissues, can cause damage to local organs as well as induce systemic damage and multiple organ failure. This study aimed to assess the potential of N-acetylcysteine (NAC) and simvastatin combination therapy in IRI prevention and treatment by assessing the level of muscle tissue malondialdehyde (MDA) and histopathological structures of gastrocnemius muscles of in animal model. Twenty-seven male Wistar strain white rats (*Rattus norvegicus*) were divided into three groups: (1) control group (P1); (2) treatment group that received oral allopurinol (P2); and (3) treatment group that received NAC andsimvastatin (P3). The levels of MDA and histopathology scores of gastrocnemius muscle were evaluated on the 4th day after intervention. The study found the mean level of MDA on gastrocnemius muscle was 70.56±3.06 nmol/mg in P1, 11.94±0.69 nmol/mg in P2, and 7.91±0.62 nmol/mg in P3. ANOVA test showed a significant difference among the groups (p=0.001). Further analysis with Tamhane test showed a significant difference in MDA level between P1 and P2 (p=0.001), P1 and P3 (p=0.001), and P2 and P3 (p=0.001). Mann-Whitney test on histopathology score showed no significant difference between P1 and P2 (p=0.902), P1 and P3 (p=0.837), and P2 and P3 (p=0.681). In conclusion, combined therapy of NAC and simvastatin significantly reduced MDA levels in animal models with IRI, although no significant difference of histopathology score was observed.

Keywords: Ischemic/reperfusion injury, N-acetylcysteine, simvastatin, malondialdehyde

Introduction

Ischemic/reperfusion injury (IRI) is defined as the damage caused by the restoration of blood supply to previously ischemic tissues.[1] Ischemia occurs when the blood and oxygen supply are less than the demand, results in cellular damage. Reestablishment of blood flow is important to save ischemic tissue; however, a sudden increase in blood and oxygen flow, known as reperfusion, induces the activation of inflammatory process and cytokines release, results in further celldamages.[2] Acute vascular occlusion (e.g., ischemic cerebral stroke, myocardial infarction, and limb ischemic treated with thrombolysis), reperfusion prevention strategy (thrombolytic therapy, angioplasty, revascularization surgeries), routine surgical procedures that cause tissue ischemia (e.g., organ transplant, free tissue transfer, vascular and cardiopulmonary bypass, use of tourniquet), major trauma, and shock are some conditions that can cause IRI.[3, 4] IRI occurs in a wide range of organs such as the heart, lung, brain, kidney, gut, and skeletal muscles;[1, 2] however, severe IRI may further induce systemic damage

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including systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS).[1, 2, 5]

In addition to interrupting cellular homeostasis that could damage cells, IRI would also cause oxidative stress and further injure organs by generating free radicals, such as reactive oxygen species.[6] Reactive oxygen species (ROS), previously known as oxygen-derived free radicals, have been suggested as potential mediators of reperfusion injury.[7] Appropriate amount of ROS is important in immune response against pathogens; however, excessive ROS [e.g., hydroxyl (OH), superoxide (O2), nitric oxide (NO), and hydrogen peroxide (H_2O_2)] can destroy organelle, cell membrane, and nuclei DNA, resulting in severe cell damages.[8] ROS damage polyunsaturated fatty acids (PUFA) of the lipid membrane, causes lipid peroxidation resulting in loss of membrane osmotic ability, which leads to oedema and cell death.[9] Malondialdehyde (MDA), a highly toxic and potentially mutagenic aldehyde, is the main and most studied product of PUFA.[10] The level of MDA in tissues can be used as a biomarker to estimate the degree of lipid peroxidation,[11] as well as to measure the level of oxidative stress.[10]

Allopurinol has been widely used as a treatment of IRI. Allopurinol, a xanthine oxidase inhibitor, prevents the formation of free radicals after reperfusion and reoxygenation of previously ischemic tissues by inhibiting the conversion of hypoxanthine.[12] Furthermore, a study showed that oral administration of allopurinol could reduce serum potassium levels as a marker of IRI on animal models.[13]In contrary, an in vivo study showed that allopurinol was effective in inhibiting xanthine oxidase activity, but did not affect lipid peroxidation.[14] Moreover, administration of allopurinol increased the risk of kidney failure up to 3.3 times, increased the risk of serious side effects such as Stevens-Johnson Syndrome and toxic epidermal necrolysis, particularly in patients with kidney problems.[15, 16] In severe IRI cases where shock, multi trauma, or kidney failure occur, the administration of allopurinol is not recommended. Thus, alternative treatment for IRI is warrant.

Administration of antioxidants, anti-thromboxane, anti-leukotrienes, and anti-platelet activating factors were suggested to prevent IRI.[17] N-acetylcysteine (NAC) is an antioxidant that play an important role in increasing intracellular glutathione levels, as well as acting as a scavenger of ROS.[18] Simvastatin showed a promising potential to alleviate IRI by inhibiting the formation of cytokines, inducing down regulation of adhesion molecules, and decreasing nitric oxide production.[19-21] These findings suggested the potential of NAC and Simvastatin for IRI treatment. This study aimed to assess the potential of combined N-acetylcysteine (NAC) and simvastatin therapy in IRI prevention and treatment by assessing the level of MDA and histopathological characteristics of muscle tissues.

Methods

Animals

Twenty-seven male Wistar strain white rats (*Rattus norvegicus*), aged 3-4 months and weighing 160-240 gram, were randomly assigned into three groups of nine: (1) control group received the intervention but did not receive any treatment, (P1); (2) treatment group that received oral allopurinol (P2); and (3) treatment group that received oral simvastatin + intraperitoneal NAC for three days post intervention (P3). One week prior to the intervention, the rats went through acclimatization process at the Animal Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya under laboratory conditions (humidity of 70-80%, and adequate lightning) and were fed and given water ad libitum. This study was conducted in

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accordance with the Guide for the Care and Use of Laboratory Animals and the Principles of Laboratory Animal Care.

Experimental procedure

A rubber band was placed on the proximal of the right and left hindlimbs of the mice for 4 h (ischemic period), followed by 72 h reperfusion period after removal of the rubber band.[20, 22] In the control group (P1), the mice were injected 0.5 cc sodium chloride (NaCl) 0.9% intraperitoneally for three days, started from the day of intervention. In the second group (P2), the mice were given 20 mg/kg/day of oral allopurinol from the day of intervention to the third day post intervention. The mice in the third group (P3) were given intraperitoneal injection of NAC (150 mg/kg/day) from the day of intervention until the third day plus 5 mg/kg/day of oral allopurinol, started from six days prior to the intervention until three days after intervention. The mice were sacrificed on the 4th day, after 72 h of reperfusion, and gastrocnemius muscle tissues were harvested for analysis.

Histopathological examination

Samples from right hindlimbs were washed with NaCl 0.9% solution and fixed with 10% neutral formaldehyde solution for 24 h. The specimens were sectioned at 5-7 μm and stained with hematoxylin and eosin before subjected to histopathological examination by light microscopy. Histopathological scoring was determined by an anatomical pathologist based on infiltration of mononuclear cells, polymorphonuclear (PMN) inflammatory cells, muscle necrosis, and bleeding as well as the severity of tissue damage.[23]

Malondialdehyde (MDA) level

Tissue samples, 1 gram, from left hindlimbs were crushed and processed with 15% TCA solution and 0.37% TBA. MDA level of the tissue was measured with MDA assay kit and the absorbance was measured using a spectrophotometer using a λ 532 nm.

Statistical Analysis

The level of MDA and histopathological scores were presented as the mean ± standard deviation (SD). The difference of MDA levels among the groups were compared using analysis of variance (ANOVA) while the difference of mean histopathology score was analyzed using Mann-Whitney test. All analyses were done using SPSS 25 version.

Results

The level of MDA

This study was conducted at Biochemistry and Pathology Anatomy Laboratories, Faculty of Medicine, Airlangga University from 2 January to 31 March 2021. Throughout the study, two mice from P1 and P2, respectively died and were dropped out of the study, left seven mice in P1 and P2 and nine mice in P3.

The study found that P3 group (NAC + simvastatin) had the lowest level of MDA among all the groups (7.91±0.62 nmol/mg). The level of tissue MDA in P2 group (allopurinol) was lower than control group (P1), but still higher than P3(Table 1).

Table 1. The level of MDA in all groups

Group	n	MDA level (mean± SD)	Minimum	Maximum
P1(control)	7	70.56 ± 3.06	65.73	73.64
P2 (allopurinol)	7	11.94 ± 0.69	10.92	12.87
P3 (NAC + simvastatin)	9	7.91 ± 0.62	7.03	9.32

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Analysis with ANOVA showed that the difference of MDA level among the three groups was significant (p=0.001). Further analysis with Tamhane test showed that the level of MDA between P1 and P2, P1 and P3, as well as P2 and P3 were significantly different (Table 2).

Table 2. Analysis of difference in MDA level among the groups

Variable	ANOVA	Multiple comparisons (Tamhane)			
	p-value	Group I	Group II	Mean difference (I-II)	p-value
MDA level (nmol/mg)	0.001	P1	P2	58.62	0.001
			P3	62.66	0.001
		P2	P1	-58.62	0.001
			P3	4.03	0.001
		P3	P1	-62.66	0.001
			P2	-4.03	0.001

Histopathology examination

Histopathology examination showed similar macroscopic and microscopic pictures in all groups. Macroscopic picture showed necrosis with hemorrhagic foci on gastrocnemius muscle, while microscopic picture showed oedema, congestive, mononuclear cells infiltration, necrosis, and bleeding in the muscle tissue (Figure 1).

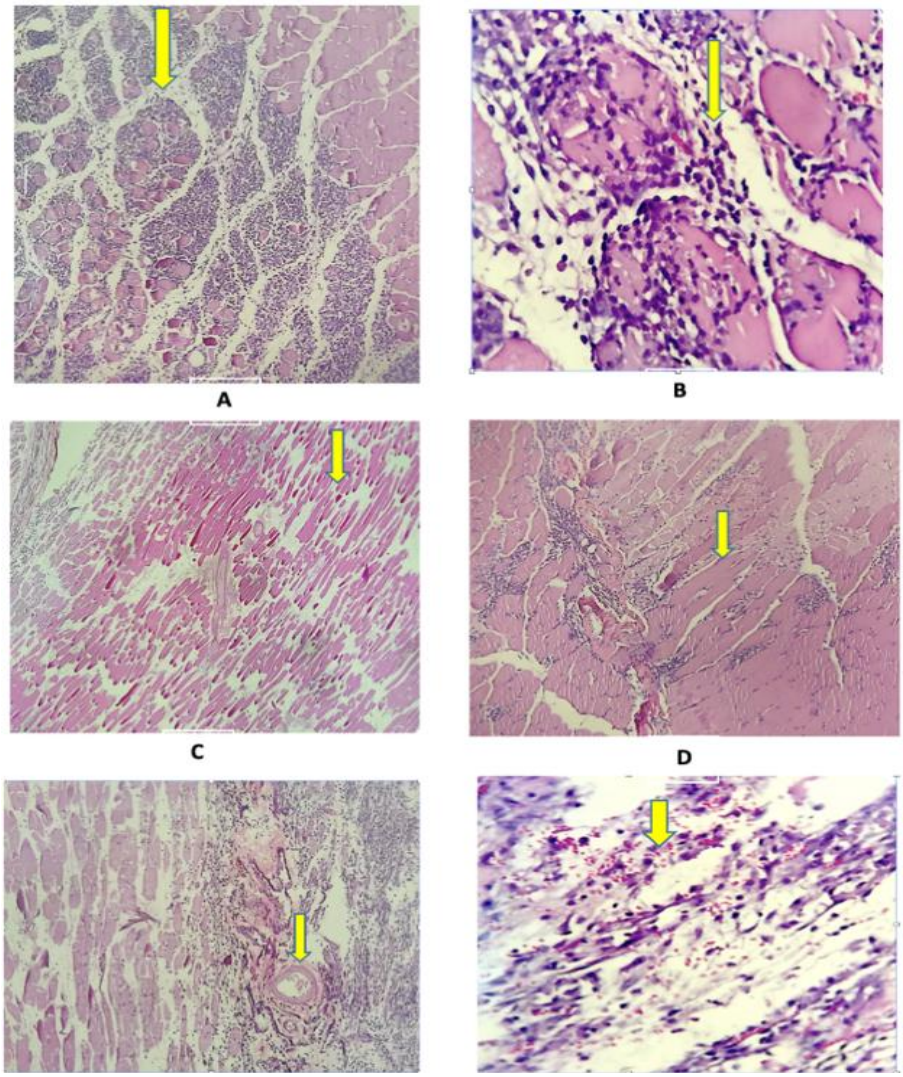

Figure 1. Microscopic picture of IRI on gastrocnemius muscle of the mice. P1 (control group): An infiltration of multifocal mononuclear inflammatory cells on 40x magnification (**A**) and 400x

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magnification (**B**). P2 (allopurinol group): tissue edema on 40x magnification (**C**), multifocal necrosis of the muscle on 400x magnification (**D**). P3 (NAC + simvastatin group): blood vessels congestion on 40x magnification (**E**), hemorrhagic area on 400x magnification (**F**).
A Kruskal-Wallis test was done to analyze the difference among total histopathological score from the three groups, as the data in P2 group was not normally distributed. Analysis showed no significant difference in total histopathology score among the groups (p=0.909). Further analysis to see the difference in median of histopathology score between pairs of groups also showed no significant differences (p=0.902 between P1 and P2; p=0.837 between P1 and P2; and p=0.681 between P2 and P3) (Table 3).

Table 3. Histopathological score differences of gastrocnemius muscle tissue from the three groups

Group	Total histopathology score	Kruskal-Wallis	Mann-Whitney		
		p-value	Group I	Group II	p-value
P1	11.86	0.909	P1	P2	0.902
P2	11.29		P1	P3	0.837
P3	12.67		P2	P3	0.681

Discussion

This study assessed the level of tissue MDA and histopathological score of animal models with IRI after the administration of combined NAC and simvastatin therapy. The study found that the mean MDA tissue level in P3 group (NAC + simvastatin group) was significantly lower than P1 (control group) and P2 (allopurinol group) (p=0.001). As the principal and most studied product of ROS, the level of MDA has been widely used as a biomarker of lipid peroxidation[11] and oxidative stress.[10] Naturally, MDA level increases significantly 2 hours post ischemic period, and will gradually decrease 24 h after reperfusion period until seven days post ischemic.[24] From this study we could see that after 72 h the MDA level in P3 (the group that was given NAC + simvastatin) was significantly lower than the control group in which the MDA level decreased naturally. The MDA level in P2, who was given Allopurinol was also significantly lower than the control group, however, still higher than the P3. Further analysis showed that the MDA level in P3 was significantly lower than P2 (p=0.001), indicating that combined therapy of NAC and Simvastatin could significantly reduce the level of MDA, even compared to the current IRI therapy Allopurinol.

The finding of this study is corresponding with previous studies that showed NAC reduced MDA levels in IRI.[25, 26] NAC is one of antioxidants and glutathione precursor that could attenuate ROS.[18, 27] Moreover, NAC inhibits inflammation signaling and expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and transforming growth factor β (TGF- β) and interleukin-1β (IL-1β), IL-6 and IL-8in cells macrophage.[28] Administration of NAC significantly reduced serum level of proinflammatory biomarkers such as IL-6, sICAM-1, NO, MDA, and neuron-specific enolase (NSE) while significantly increased serum level of antioxidant biomarkers such as glutathione peroxidase (GPx), total thiol groups (TTG) and superoxide dismutase (SOD).[29]

In addition to NAC, the mice in P3 were also given simvastatin. Previous studies suggested potential of Simvastatin as IRI treatment by inhibiting the formation of cytokines, inducing down regulation of adhesion molecules, and decreasing nitric oxide production.[20, 21] Another study suggested that Statin could reduce vascular permeability in animal models with cardiac and lung IRI.[19, 30] In this study, the mice treated withsimvastatin as a pre-treatment.

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Investigation showed that pre-treatment with simvastatin could reduce the accumulation of leukocyte and vascular permeability in animal models with ischemic lung injury.[30] Large amount of free radicals is released at the early phase of IRI, causing irreversible damage to the cells.[31] Therefore, pre-treatment is necessary to prevent the excessive release of ROS and further prevent irreversible damage to the cells. Combined therapy of NAC and simvastatin in this study was expected to give better result, as shown by significantly lower MDA level.

No significant difference in histopathological score was found among the three groups in this study. A previous study suggested that IRI lasting for more than 2 h caused extensive skeletal muscle damage, and although skeletal muscle is able to regenerate, this process takes several weeks. Another study reported that full recovery of muscle after 4 h of ischemia was not reached even after 14 days.[32]It has been documented that dead muscle fibers had not yet disappeared on the 14th day after IRI, following a period of 3 h of ischemia in mice.[22] These explained why no significant difference in histopathological appearance and score were observed in this study.

This study, however, is subject to several limitations. First, this study only assessed the level of MDA as one of the ROS. In fact, other ROS products such as hydroxyl (OH), superoxide (O2), hydrogen peroxide (H_2O_2), and nitric oxide (NO) might also cause cell damage during IRI. Thus, a study that assess these markers in the future is important. Second, short period of observation time did not allow this study to elucidate the effect of NAC and Simvastatin on muscle recovery following IRI. A study with longer observation time to assess muscle recovery following IRI is warrant in the future. Nonetheless, the finding of this study highlights the potential of NAC and Simvastatin in IRI treatment by reducing the level of MDA in muscle tissue.

Conclusions

Combined therapy of NAC and simvastatin significantly reduced the level of MDA in animal models with IRI, suggesting its potential for IRI treatment and prevention. However, no significant difference of histopathological appearance or scoring were found in animal models received combined NAC and simvastatin therapy.

References

1. Cowled P, Fitridge R. Pathophysiology of Reperfusion Injury. In: Fitridge R, Thompson M, editors. Mechanisms of Vascular Specialists [Internet]. Adelaide (AU): University of Adelaide Press; 2011.
2. Ikhlas M, Atherton NS. Vascular Reperfusion Injury. StatPearls. Treasure Island (FL)2021.
3. Dong X, Xing Q, Li Y, Han X, Sun L. Dexmedetomidine protects against ischemia-reperfusion injury in rat skeletal muscle. J Surg Res. 2014; 186:240-5.
4. Dorweiler B, Pruefer D, Andrasi TB, Maksan SM, Schmiedt W, Neufang A, et al. Ischemia-Reperfusion Injury : Pathophysiology and Clinical Implications. Eur J Trauma Emerg Surg. 2007; 33:600-12.
5. Chen W, Li D. Reactive Oxygen Species (ROS)-Responsive Nanomedicine for Solving Ischemia-Reperfusion Injury. Front Chem. 2020; 8:732.
6. Zhang W, Wang M, Xie HY, Zhou L, Meng XQ, Shi J, et al. Role of reactive oxygen species in mediating hepatic ischemia-reperfusion injury and its therapeutic applications in liver transplantation. Transplant Proc. 2007; 39:1332-7.
7. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biology. 2015; 6:524-51.

8. Nita M, Grzybowski A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. Oxid Med Cell Longev. 2016; 2016:3164734.
9. Gillani S, Cao J, Suzuki T, Hak DJ. The effect of ischemia reperfusion injury on skeletal muscle. Injury. 2012; 43:670-5.
10. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis. 2005; 15:316-28.
11. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014; 2014:360438.
12. Pacher P, Nivorozhkin A, Szabó C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol Rev. 2006; 58:87-114.
13. Prasetyanto D. Efek Allopurinol per-oral dalam mengurangi kerusakan seluler akibat reperfusion injury pada iskemia tungkai kelinci (*Oryctolagus cuniculuc*) yang dilakukan tindakan reperfusi. Surabaya: Universitas Airlangga; 2012.
14. Fields M, Lewis CG, Lure MD. Allopurinol, an inhibitor of xanthine oxidase, reduces uric acid levels and modifies the signs associated with copper deficiency in rats fed fructose. Free Radic Biol Med. 1996; 20:595-600.
15. Rey A, Batteux B, Laville SM, Marienne J, Masmoudi K, Gras-Champel V, et al. Acute kidney injury associated with febuxostat and allopurinol: a post-marketing study. Arthritis Res Ther. 2019; 21:229.
16. Erdinc M, Erdinc L, Nergiz Y, Isik B. Potentiation of cisplatin-induced nephrotoxicity in rats by allopurinol. Exp Toxicol Pathol. 2000; 52:329-34.
17. Wang WZ, Baynosa RC, Zamboni WA. Therapeutic interventions against reperfusion injury in skeletal muscle. J Surg Res. 2011; 171:175-82.
18. Aksu V, Yuksel V, Chousein S, Tastekin E, Iscan S, Sagiroglu G, et al. The effects of sildenafil and n-acetylcysteine on ischemia and reperfusion injury in gastrocnemius muscle and femoral artery endothelium. Vascular. 2015; 23:21-30.
19. Di Napoli P, Antonio Taccardi A, Grilli A, Spina R, Felaco M, Barsotti A, et al. Simvastatin reduces reperfusion injury by modulating nitric oxide synthase expression: an ex vivo study in isolated working rat hearts. Cardiovasc Res. 2001; 51:283-93.
20. Cowled PA, Khanna A, Laws PE, Field JB, Varelias A, Fitridge RA. Statins inhibit neutrophil infiltration in skeletal muscle reperfusion injury. J Surg Res. 2007; 141:267-76.
21. Yan Y, Lv X, Ma J, Hong G, Li S, Shen J, et al. Simvastatin Alleviates Intestinal Ischemia/Reperfusion Injury by Modulating Omi/HtrA2 Signaling Pathways. Transplant Proc. 2019; 51:2798-807.
22. Vignaud A, Hourde C, Medja F, Agbulut O, Butler-Browne G, Ferry A. Impaired skeletal muscle repair after ischemia-reperfusion injury in mice. J Biomed Biotechnol. 2010; 2010:724914.
23. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. Nat Rev Immunol. 2016; 16:378-91.
24. Arato E, Kurthy M, Sinay L, Kasza G, Menyhei G, Hardi P, et al. Effect of vitamin E on reperfusion injuries during reconstructive vascular operations on lower limbs. Clin Hemorheol Microcirc. 2010; 44:125-36.
25. Takhtfooladi HA, Hesaraki S, Razmara F, Takhtfooladi MA, Hajizadeh H. Effects of N-acetylcysteine and pentoxifylline on remote lung injury in a rat model of hind-limb ischemia/reperfusion injury. J Bras Pneumol. 2016; 42:9-14.
26. Talasaz AH, Khalili H, Fahimi F, Mojtaba S. Potential role of N-acetylcysteine in cardiovascular disorders. Therapy. 2011; 8:237-45.
27. Sun Y, Pu LY, Lu L, Wang XH, Zhang F, Rao JH. N-acetylcysteine attenuates reactive-oxygen-species-mediated endoplasmic reticulum stress during liver ischemia-reperfusion injury. World J Gastroenterol. 2014; 20:15289-98.
28. Pei Y, Liu H, Yang Y, Yang Y, Jiao Y, Tay FR, et al. Biological Activities and Potential Oral Applications of N-Acetylcysteine: Progress and Prospects. Oxid Med Cell Longev. 2018; 2018:2835787.

29. Sabetghadam M, Mazdeh M, Abolfathi P, Mohammadi Y, Mehrpooya M. Evidence for a Beneficial Effect of Oral N-acetylcysteine on Functional Outcomes and Inflammatory Biomarkers in Patients with Acute Ischemic Stroke. Neuropsychiatr Dis Treat. 2020; 16:1265-78.
30. van de Visse EP, van der Heijden M, Verheij J, van Nieuw Amerongen GP, van Hinsbergh VW, Girbes AR, et al. Effect of prior statin therapy on capillary permeability in the lungs after cardiac or vascular surgery. Eur Respir J. 2006; 27:1026-32.
31. Kumar V, Abbas A, Aster J. Robbins Basic Pathology. 10th ed. Philadelphia: Elsevier; 2017.
32. Walters TJ, Kragh JF, Kauvar DS, Baer DG. The combined influence of hemorrhage and tourniquet application on the recovery of muscle function in rats. J Orthop Trauma. 2008; 22:47-51.