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Research Article

Comparative evaluation between ascorbic acid and N-acetyl cysteine for preventing tourniquet induced ischaemic reperfusion injury during lower limb surgery, a randomized controlled trial

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KEYWORDS

Ascorbic acid;
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Abstract *Background:* During tourniquet induced ischemic reperfusion, reactive oxygen species and cytokines appear and cause cellular damage and remote tissue injury.
Aim: To compare the effects of preoperative intravenous infusion of N-acetyl cysteine or ascorbic acid on the production of malonyldialdehyde as a marker of oxidative stress and IL-6 and IL-8 as markers of systemic inflammation after ischemic reperfusion as primary outcomes. Also both agents were compared regarding post-deflation hemodynamic effects and post-deflation changes in arterial pH and lactic acid as secondary outcomes.
Patient and methods: 60 patients, scheduled for unilateral lower extremity surgery with a pneumatic tourniquet, were included. The study was designed as a randomized controlled parallel arms superiority trial. Baseline collection of blood samples was done and then patients were randomly classified into three groups: Group A; received 1 g Ascorbic acid, group N; received 10 mg/kg NAC and group C (control group): received 100 ml saline infusion. Epidural anesthesia was administrated. Two blood samples were drawn at each assessment time. One sample for arterial blood pH and lactic acid measurement and the other blood sample was centrifuged and stored at −20 °C for subsequent analysis of MDA, IL-6 and IL-8.
Results: Levels of MDA, IL-6, and IL-8 were significantly increased in group C after tourniquet release compared with the baseline. In groups A and N, MDA did not increase over baseline values, but IL-6 and IL-8 levels were increased and their levels were significantly less than in the control group.

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Changes in hemodynamics, pH and serum lactate were more evident in group C than groups A and N.
Conclusion: Both N-acetyl cysteine and ascorbic acid reduce post-deflation increase in blood levels of markers of oxidative stress and markers of systemic inflammation and thus both are beneficial in preventing post-tourniquet deflation ischemic reperfusion injury in lower limb surgery.

1. Introduction

Application of proximal pneumatic tourniquet is widely used in extremity surgeries to control intraoperative bleeding. Ischemia occurs following tourniquet inflation, and reperfusion occurs after deflation because of the re-establishment of blood flow [1]. The hemodynamic and metabolic effects that occur with tourniquet application depend on the duration of tourniquet placement, size of the ischemic area, type of anesthesia, and the cardiovascular condition of the patient [2]. Sequestration of oxygenated blood cells in the extremity during ischemia leads to the appearance of free oxygen radicals [3]. With the event of reperfusion, the Reactive Oxygen Species (ROS) and cytokines appear and cause damage to cellular structures through the process of the lipid peroxidation of cellular membranes and macromolecules [4]. The Cellular damage that occurs after reperfusion of previously viable ischemic tissue is defined as ischemic reperfusion (IR) injury [5]. Antioxidant medications and/or cytokine-modulating therapies might, therefore, reduce morbidity [6]. Lipid peroxidation represents a chain reaction leading to oxidation of polyunsaturated fatty acids, disrupting membrane structure and producing toxic metabolites such as malonyldialdehyde (MDA) which is used as a sensitive marker of ischemic reperfusion injury [7]. Interleukin 6 (IL-6), which is reliable indicator of systemic inflammation, also correlates with the extent of reperfusion injury [8]. IL-6 has been correlated with the activation of circulating neutrophil and potential tissue injury [9]. Circulating IL-8 has also been implicated with the process of lower limb ischemia–reperfusion injury which may cause neutrophil sequestration and increased microvascular permeability especially in the lung [10]. N-acetyl cysteine (NAC), an n-acetyl derivative of the amino acid L-cysteine, is a thiol-containing compound which has been widely used for many years as a mucolytic drug in the treatment of chronic obstructive pulmonary disease. It also has antioxidant, cytoprotective and microcirculatory effects [11]. Its pharmacological actions include restoration of cellular antioxidant potential by restoration of depleted glutathione, direct scavenging of oxygen-free radicals, and inhibition of neutrophil activity and TNF production [12]. Vitamin C, also known as ascorbic acid, is a water-soluble antioxidant molecule that removes the aqueous phase oxygen free radicals by a rapid electron transfer and inhibits oxidative damage to important biological macromolecules, including DNA, proteins, and lipids [13].

In this prospective, double blind, placebo controlled study we compared the effects of preoperative intravenous infusion of N-acetyl cysteine or Ascorbic acid on the production of MDA as a marker of oxidative stress and IL-6 and IL-8 as markers of systemic inflammation after tourniquet induced ischemic reperfusion injury during lower limb surgery as primary outcomes. Also we compared both agents regarding post-deflation hemodynamic effects by comparing total doses of ephedrine consumed and post-deflation changes in arterial pH and lactic acid levels as secondary outcomes.

2. Patients and methods

After approval of the hospital ethics committee and written informed consent, 60 patients ASA physical status I or II, aged 25–45 years, scheduled for unilateral lower extremity surgery with a pneumatic tourniquet for a duration of 60–90 min, were included in this study. Exclusion criteria included the presence of vascular disease, cardiorespiratory disease, renal insufficiency, liver dysfunction, diabetes or the administration of any antioxidant or anti-inflammatory drug.

No premedication was given. Warm fluids were given to control the mean arterial blood pressure and temperature within normal ranges throughout the study. The study was designed as a randomized controlled parallel arms superiority trial. Baseline collection of blood samples (T0) was done and then sealed, and opaque envelopes containing a computer-generated random sequence were used for randomization of three groups. Randomization type was simple and the randomization code was concealed until the end of the trial and analysis of data.

Group A (Ascorbic acid group): $N = 20$, received 1 g vitamin C (Ascorbic acid) dissolved in 100 mL 0.9% normal saline intravenously infused within one hour. Group N (N-acetyl cysteine group): $N = 20$ N received 10 mg/kg NAC dissolved in 100 mL 0.9% normal saline intravenously infused within one hour and Group C (control group): $N = 20$ received the same volume of saline (100 mL) infused intravenously within one hour as a placebo.

After infusion of the study drugs, epidural anesthesia was administrated at the L2–L3 or L3–L4 interspace using 0.5% bupivacaine. The sensory block level was kept below T10 (in order to minimize the hypotensive effect of epidural anesthesia as possible). The lower extremity was exsanguinated and a pneumatic tourniquet was inflated to a pressure of approximately twice the systolic blood pressure. All operations were performed in the supine position.

Intraoperative arterial blood pressure values of 20% less than basal were considered hypotensive. Intravenous bolus (IV) ephedrine 3 mg was administered as a bolus in the setting of hypotension followed by 1 mg increments to restore baseline blood pressure. When intraoperative bleeding occurred, volume replacement was initiated with saline instead of blood products. The patient demographics, the tourniquet times, volume of local anesthetic and the dosages of ephedrine were recorded. Two blood samples were drawn at each assessment time, at baseline (T0), 1 min after tourniquet placement (T1), 1 min before tourniquet release (T2), 5 min after tourniquet release (T3), 20 min ATR (T4) and 1 h ATR (after tourniquet

Table 1 Demographic and operative data.

Variable	Group A ($N = 20$)	Group N ($N = 20$)	Group C ($N = 20$)	p-value
Male/Female	11/9	10/10	11/9	0.935
Age (years)	36 (6)	35 (8)	35 (7)	0.875
BMI (kg/m^2)	23.8 (2.7)	24.2 (2.5)	23.9 (2.6)	0.880
Surgical procedure				0.360
Fixation of tibial fracture	8 (40%)	8 (40%)	9 (45%)	
Fixation of ankle fracture	8 (40%)	9 (45%)	8 (40%)	
Knee arthroscopic	4 (20%)	3 (15%)	3 (15%)	
Local anesthetic volume (ml)	12.5 (1.4)	12.4 (1.5)	12.3 (1.3)	0.904
Level of sensory block T11 (T10–T12)	20 (100%)	20 (100%)	20 (100%)	0.958
Tourniquet time (min)	74 (13)	75 (14)	77 (11)	0.751

Data are presented as ratio, mean (SD), number (%), or median (interquartile range).
BMI, body mass index.

Table 2 Assays of plasma malonyldialdehyde and serum interleukins levels.

Variable	Time	Group A ($n = 20$)	Group N ($n = -20$)	Group C ($n = -20$)	p-value
MDA (nmol/l)	T0	2.9 (0.7)	3.0 (0.8)	2.8 (0.9)	0.735
	T1	3.0 (0.6)	3.0 (0.7)	2.9 (0.8)	0.875
	T2	2.7 (0.9)	2.8 (0.8)	2.8 (0.8)	0.909
	T3	3.0 (0.7)	2.9 (0.8)	6.1 (0.4)[a]	<0.0001
	T4	2.9 (0.7)	3.0 (0.8)	5.8 (0.8)[a]	<0.0001
	T5	2.9 (0.7)	3.0 (0.8)	5.5 (0.8)[a]	<0.0001
IL-6 (pg/ml)	T0	2.7 (0.8)	2.8 (0.7)	2.9 (0.7)	0.692
	T1	3.4 (0.7)	3.3 (0.6)	3.4 (0.6)	0.848
	T2	3.9 (0.60	4.1 (0.4)	4.0 (0.5)	0.464
	T3	6.8 (0.5)	6.7 (0.6)	12.3 (0.8)[a]	<0.0001
	T4	6.4 (0.7)	6.5 (0.9)	13.2 (0.8)[a]	<0.0001
	T5	5.3 (0.6)	5.4 (0.8)	13.3 (0.6)[a]	<0.0001
IL-8 (pg/ml)	T0	11.6 (0.7)	11.7 (0.6)	11.8 (0.8)	0.670
	T1	11.5 (0.7)	11.5 (0.6)	11.4 (0.6)	0.848
	T2	11.7 (0.5)	11.3 (0.6)	11.0 (0.5)	0.0005
	T3	16.8 (0.80	16.7 (0.6)	32.3 (0.7)[a]	<0.0001
	T4	15.4 (0.6)	15.5 (0.7)	33.2 (0.7)[a]	<0.0001
	T5	15.2 (0.5)	14.9 (0.6)	33.6 (0.6)[a]	<0.0001

Data are presented as mean (SD).
IL-6, interleukin-6; IL-8, interleukin-8; MDA, malonyldialdehyde; T0, baseline (T0); T1, 1 min after tourniquet placement; T2, 1 min before tourniquet release; T3, 5 min after tourniquet release; T4, 20 min after tourniquet release; T5, 1 h after tourniquet release.
[a] p-value < 0.001 vs. Group A and Group N.

release) (T5). One sample for Arterial blood pH and serum lactate measurement and the other blood sample were centrifuged to separate the serum and plasma, which were then stored at −20 °C for subsequent analysis of MDA, IL-6 and IL-8.

The plasma MDA was analyzed by the method of thiobarbituric acid reaction (Jiancheng Bio-engineering Research Institute, Nanjing, China); IL-6 and IL-8 were determined using enzyme linked immunosorbent assay with kits (Jingmei Sciences Co., Ltd., Shenzhen, China) according to manufacturer's instructions.

To maintain the double blind nature of the study, an anesthesia resident who was not present during the patients' group allocation and tourniquet application collected data and analyzed blood gas. Circulating mediators were analyzed by investigators who were unaware of the patients' group assignment and the blood gas results.

3. Statistical methods

Statistical analysis was done using GraphPad© Prism© for Windows© version 6 and GraphPad© Instat© version 3.05 (GraphPad Software, Inc., San Diego California, USA).

It was estimated that a sample of 20 patients per group would have a power of 86% to detect a large effect size of $f = 0.45$ as regards the primary outcome (production of malonyldialdehyde, IL-6 and IL-8) measures using the F-test and assuming a two-sided type I error of 0.05.

Continuous numerical variables were presented as mean (SD) and intergroup comparisons were done using one-way analysis of variance (ANOVA) with the use of the Tukey–Kramer test for post hoc comparisons. Discrete variables were presented as median (interquartile range) and the Kruskal–Wallis test was used for comparison of the groups. Categorical

Table 3 Total dose of ephedrine.

Variable	Group A ($N = 20$)	Group N ($N = 20$)	Group C ($N = 20$)	p-value
Total dose of ephedrine before tourniquet release (mg)	10.2 (3.3)	9.9 (4.1)	10.1 (3.8)	0.967
Total dose of ephedrine after tourniquet release (mg)	4.3 (1.4)	4.1 (1.5)	7.6 (2.0)[a]	<0.0001

Data are presented as mean (SD).
[a] P-value < 0.001 versus Group A and Group N.

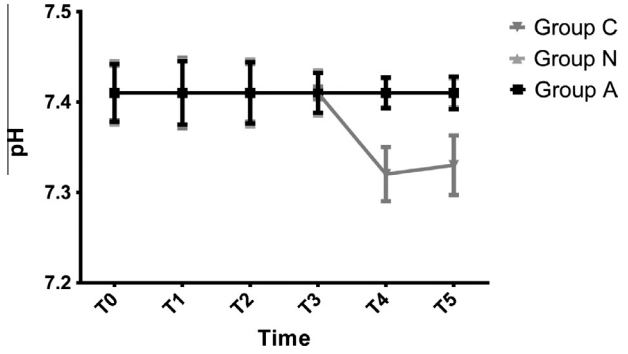

Figure 1 Changes in pH in the three study groups. Error bars represent SD. T0, baseline (T0); T1, 1 min after tourniquet placement; T2, 1 min before tourniquet release; T3, 5 min after tourniquet release; T4, 20 min after tourniquet release; T5, 1 h after tourniquet release.

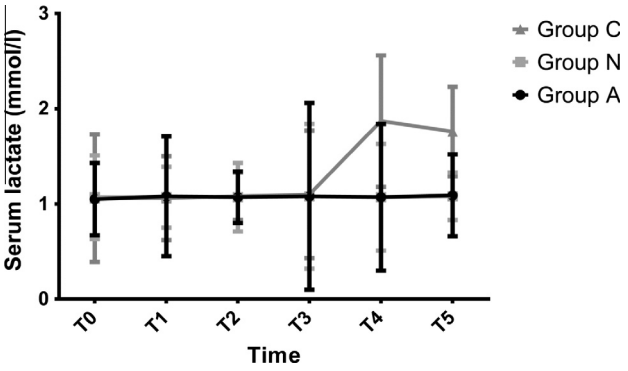

Figure 2 Changes in serum lactate in the three study groups. Error bars represent SD. T0, baseline (T0); T1, 1 min after tourniquet placement; T2, 1 min before tourniquet release; T3, 5 min after tourniquet release; T4, 20 min after tourniquet release; T5, 1 h after tourniquet release.

data were presented as ratio or as number (%) and the Pearson chi square test was used to test between-group differences.

4. Results

All 60 patients who were included completed the study. As is shown in Table 1, there were no differences between the 3 groups as regards the demographic data. The types of surgical procedures were comparable in the 3 groups as well as were the tourniquet time, the upper level of sensory block, and the volume of local anesthetic used.

In the control group (C), MDA, IL-6, and IL-8 levels were significantly increased at T3, T4 and T5 after release of the tourniquet compared with the baseline (p-value < 0.0001). In groups (A and N), MDA did not increase over baseline values. IL-6 and IL-8 levels were increased in both groups (A and N) at T3, T4 and T5 after release of the tourniquet, but their levels were significantly less than in the control group (Table 2).

There was no difference between the three groups comparing the total dose of ephedrine used to control the blood pressure before tourniquet release, while there were significant differences between the three groups as regards the total dose of ephedrine used to control blood pressure after tourniquet release (ATR) (p-value < 0.0001) (Table 3). In group C there was more consumption of ephedrine than the other two groups.

Figs. 1 and 2 show pH and lactic acid values in the 3 groups at different observation times. There were a decrease in pH and an increase in serum lactate at T4 and T5 only in group (C) when compared with the other 2 groups.

5. Discussion

The aim of our study was to compare the effects of preoperative intravenous infusion of N-acetyl cysteine or Ascorbic acid on the production of MDA as a marker of oxidative stress and IL-6 and IL-8 as markers of systemic inflammation after tourniquet induced ischemic reperfusion injury during lower limb surgery as primary outcomes. Also we compared both agents regarding post-deflation hemodynamic effects by comparing total doses of ephedrine consumed and post-deflation changes in arterial pH and serum lactate as secondary outcomes.

During tourniquet induced ischemia, cells cannot maintain their membrane integrity, which releases calcium and phospholipid A2 as well as formation of polyunsaturated fatty acids and fatty acid radicals [14]. With reperfusion, fatty acid radicals react with oxygen, triggering lipid peroxidation, which is an autocatalytic mechanism leading to oxidative destruction of cell membranes that increases membrane permeability and stimulates leukocyte chemotaxis with the production of toxic reactive metabolites and cell death [15]. Thus, oxygen derived free radicals and proteolytic enzymes are released from the cells and these materials damage the cells in remote tissues. Consequently, amino acid modifications including 8-hydroxydeoxyguanosine, carbonyls, methionine sulphoxide and MDA are produced. Those oxygen-free radicals or lipid peroxides are short lived and difficult to measure directly. MDA is a more stable degradation product of lipid peroxides, is often assayed, and is believed to reflect the lipid peroxidation level [16]. A significant increase in the level of MDA has been observed in the lung tissue and in plasma of patients

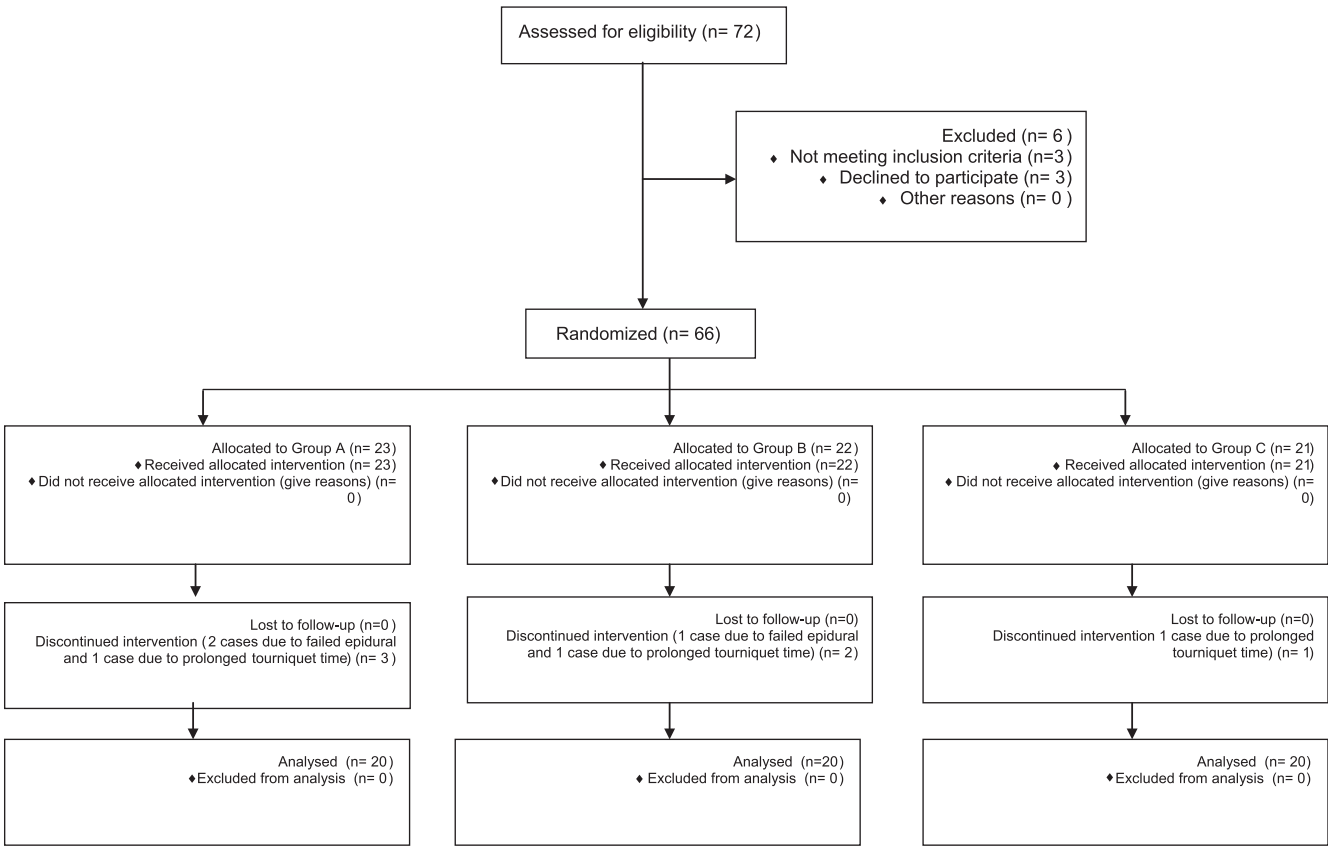

Figure 3 Participant's flow diagram.

undergoing lower limb ischemia–reperfusion [17,18]. IL-6, which is a reliable indicator of systemic inflammation, also correlates with the extent of reperfusion injury and has also been associated with the activation of circulating neutrophil and potential tissue injury [8,9]. Circulating IL-8 has also been implicated in the process of lower limb ischemia–reperfusion injury by a mechanism suggested to involve neutrophil sequestration and increased microvascular permeability in the lung [19,10]. The correlations of the increased levels of MDA, IL-6, and IL-8 with the hemodynamic and blood gas variables in our study support the hypothesis that lipid peroxidation and systemic inflammatory response may play a role in ischemic reperfusion injury after tourniquet induced lower limb ischemia.

The results of our study showed that the use of preoperative N-acetyl cysteine or ascorbic acid decreased the production of MDA, IL-6 and IL-8 during reperfusion after tourniquet induced ischemia, also during the post-inflation period, and there were more stable hemodynamics and no changes in pH or lactic acid levels with the use of these two drugs.

The reduction of the harmful effects of reperfusion injury has been investigated through different approaches including the following: anesthetic techniques [20], preconditioning [21] and drugs as ketamine [22], dexmedetomidine [23] or propofol [24].

NAC is an antioxidant which reacts strongly with the hydroxyl radical and with hypochloric acid, but reacts poorly with the hydrogen peroxide and superoxide radicals. It may also have an indirect antioxidant effect by facilitating glutathione biosynthesis and supplying GSH for glutathione peroxidase catalyzed reactions [25]. A study done by Nagasaki and co-workers showed that in glutathione-depleted liver, NAC prevents hepatic injury and improves liver integrity after I/R injury, as a direct free-radical scavenger not by acting as a substrate for glutathione synthesis [26]. Low-dose N-acetyl cysteine infusion (5 mg/kg/h) was found to attenuate lipid peroxidation and IR injury in arthroscopic knee surgery requiring tourniquet application [27]. In a study conducted by Koca and his co-workers, NAC and ischemic preconditioning were found to have protective effect on the occurrence of oxidative stress resulting from IR period by preventing MDA, super oxide dismutase glutathione peroxidase, total antioxidant capacity, and total oxidant status changes in routine arthroscopic knee surgery [28].

Vitamin C, also known as ascorbic acid, is a potent water soluble natural antioxidant that quenches ROS and inhibits ROS mediated nitric oxide inactivation. In addition, vitamin C can act as coantioxidant by regenerating other antioxidants [29].

Nightingale et al. found that in patients with cardiac failure, nitric oxide (NO) bioavailability increased in the brain stem and blood vessels after intravenous infusion of high-dose vitamin C, leading to improvements in blood vessel distensibility and baroreceptor reflex function. They also reported that this phenomenon directly prevented free oxygen radicals from suppressing the baroreceptors. Oxidative stress regulates NO metabolism in the brain stem and is, therefore, involved in baroreceptor reflex regulation. This might maintain the

vascular resistance and baroreceptor reflex and, as a result, mean arterial pressure could also be maintained with the use of high-dose vitamin C [30].

Clinically, the antioxidant effect of vitamin C has been studied in some surgical models. A study done by Dingchao et al., reported that MDA, creatinine phosphokinase (CPK-MB) and lactate dehydrogenase (LDH) levels decreased following the administration of high-dose vitamin C in patients undergoing open heart surgery which may offer some protection for cardiac muscle [31].

In a study conducted by Basili et al., they reported that in patients undergoing elective percutaneous coronary intervention, vitamin C infusion blunted in vivo the increase of 2 markers of oxidative stress, namely (8-OHdG) 8-hydroxy-2′-deoxyguanosine and 8-iso-prostaglandin F2alpha (F2-IPs) with concomitant improvement of thrombolysis in myocardial infarction (myocardial perfusion grade) [32].

Another study showed that vitamin C infusion before percutaneous coronary intervention (PCI) was associated with a lower incidence of PMI (Periprocedural myocardial injury). The investigators suggested that the cardioprotective effect of vitamin C may result from the inhibition of oxidative stress [33].

Administering high-dose vitamin C to elderly patients undergoing bilateral total knee replacement (TKR) surgery could lower MDA levels and prevent the unstable hemodynamics and hypoxia caused by IR injury in both knees, decreasing the incidence of both cardiopulmonary complication and mortality rates [34].

In conclusion, the results of our study showed that both N-acetyl cysteine and vitamin C (ascorbic acid) reduce post-deflation increase in blood levels of markers of oxidative stress and markers of systemic inflammation and thus both are beneficial in preventing post-tourniquet deflation ischemic reperfusion injury in lower limb surgery.

6. Limitations of the study

There were no limitations of the study.

Conflict of interest

No conflict of interests.

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