Effect of high-dose vitamin C on renal ischemia-reperfusion injury

Seo hee Ko

Department of Medicine

The Graduate School, Yonsei University

Effect of high-dose vitamin C on renal ischemia-reperfusion injury

Directed by Professor Jae-Kwang Shim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Science

Seo hee Ko

December 2023

This certifies that the Doctoral Dissertation of
Seo hee Ko is approved.

Thesis Supervisor: Jae-Kwang Shim

Thesis Committee Member#1: Young-Lan Kwak

Thesis Committee Member#2: Beom Seok Kim

Thesis Committee Member#3: Sung Yong Park

Thesis Committee Member#4: Hyun-Chel Joo

The Graduate School
Yonsei University

December 2023

ACKNOWLEDGEMENTS

With the completion of this dissertation, a significant chapter of my life as a student has finally come to a close. It could not be possible without the assistance and support of many people.

First and foremost, I wish to express my sincere gratitude to my doctoral supervisor, Professor Jae-Kwang Shim, for his guidance, invaluable suggestions, and patience throughout the preparation of this dissertation. It has been a great privilege and fortune to learn from him. His profound knowledge and insight have been a source of constant inspiration for my research.

Secondly, I had the opportunity to grow as a specialist under the guidance of great professors in the Department of Anesthesiology and Pain Medicine at Severance Hospital. I am eternally grateful to Professor Young-Lan Kwak and the other esteemed professors for their consistent support since my residency, enriching me with their expertise and valuable wisdoms.

Third, I extend my sincere thanks to my dissertation committee members for their precious time and expertise. Their comments and advice have been instrumental in shaping my research and will continue to inform my future work.

Lastly, my deepest gratitude and appreciate to my family for unwavering support and love, fostering my growth and resilience. Throughout my life, facing numerous challenges is inevitable but knowing I have my family’s unwavering support enables me to move forward with courage

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Effect of high-dose vitamin C on renal ischemia-reperfusion injury

Seo hee Ko

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jae-Kwang Shim)

Background Acute kidney injury (AKI) remains to be the most frequent complication after cardiac surgery exerting adverse influence on patient outcomes. Major cogent underlying mechanisms of AKI is related to renal ischemia-reperfusion (I/R) injury, exacerbated by systemic inflammation associated with surgical stress and cardiopulmonary bypass (CPB). Various experimental approaches to prevent AKI through mitigating the I/R injury and inflammation have achieved limited clinical success. Vitamin C constitutes the primary innate defense mechanism against oxidative stress and has been proposed as a potential therapeutic agent for I/R injury, which cannot be internally synthesized and has been shown to be depleted in critically ill states. Therefore, it seems plausible to hypothesize that timely administration of high-dose vitamin C can mitigate the overwhelming oxidative stress, which is the main trigger for complex downstream pathophysiologic mechanisms of cardiac surgery-induced AKI, and alleviate the development of AKI. The aim of present study was to assess the effect of high-dose vitamin C in preventing renal I/R injury.

Methods The experiment was conducted in two phases: first to determine the ideal time of administration, and then the optimal dose. Renal I/R was induced by 45 minutes of ischemia (clamping of renal arteries), followed by 24 hours of reperfusion. After reperfusion, kidney tissue and biochemical markers were evaluated. Four groups of Sprague-Dawley rats were randomly assigned: sham, IRC (I/R + saline), pre-vitC (vitamin C + I/R), post-vitC (I/R + vitamin C). Vitamin C 200 mg/kg was administered intravenously at 1 h before ischemia

(pre-vitC) or upon reperfusion (post-vitC). We further assigned two more groups of Sprague–Dawley rats to test varying doses using the optimal timing previously identified: V100 (Vitamin C 100mg/kg), V300 (Vitamin C 300mg/kg). The V200 group utilized samples from the previous step. Vitamin C was administered intravenously upon reperfusion.

Results Vitamin C administration upon reperfusion ameliorated renal dysfunction and diminished tubular damage in renal tissues following renal I/R injury. Using 100 mg/kg and 200 mg/kg vitamin C during reperfusion decreased oxidative stress markers Myeloperoxidase (MPO), thioredoxin-interacting protein (TXNIP), and anti-NADPH oxidase 4 (NOX4). Furthermore, vitamin C mitigated ROS-associated inflammatory responses by decreasing HMGB1 release, IL-1β, IL-6, and TNF-α levels compared with the sham group. Vitamin C post-treatment at 100mg/kg and 200mg/kg doses favorably modulated I/R-induced increases in inflammasomes [NOD like receptor 3 (NLRP3), apoptosis-associated speck-like protein (ASC), Caspase-1]. In the 300 mg/kg vitamin C group showed similar findings to the IRC group in terms of both serum BUN and Cr levels and histological evaluation. In addition, it could not induce further improvement in oxidative stress markers and inflammatory response in I/R injury.

Conclusions High-dose vitamin C treatment at a dose of 200 mg/kg upon reperfusion could significantly mitigate the I/R injury-induced renal injury through effectively reducing oxidative stress. This effect was associated with the diminished activation of upstream triggers leading to ensuing inflammation related to HMGB1 and inflammasome activation.

Key words : vitamin C, ascorbic acid, acute kidney injury, ischemia-reperfusion injury

Effect of high-dose vitamin C on renal ischemia-reperfusion injury

Seo hee Ko

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jae-Kwang Shim)

I. INTRODUCTION

Cardiac surgery requiring extracorporeal circulation and cardioplegic arrest inevitably induces ischemia-reperfusion (I/R) injury to major organs and triggers systemic inflammatory reactions.[1-3] I/R injury of major organs following cardiac surgery represents a significant clinical challenge due to heightened risk of relevant complications. One of the most common complications is acute kidney injury (AKI) presented as extensive tubular injury, which is known to substantially increase morbidity and mortality.[4, 5] Of note, even temporary elevations in serum creatinine have been shown to increase the risk of progression to chronic kidney disease (CKD).[6, 7] Unfortunately, there are no definite treatment strategies, and the current clinical paradigm is focused on its prevention. In that context, therapies aimed at suppressing inflammation involving the use of corticosteroids conveyed contradictory and rather disappointing clinical results.[8-10] The main reason is that the systemic inflammatory reaction itself is a natural, innate mechanism to protect the body; while organ damage arises when the response is exaggerated. Currently, it is difficult to distinguish these two scenarios clinically.

Accordingly, the process of I/R injury was heavily scrutinized for potential therapeutic targets. Albeit being complex and multifactorial, one of the most prominent and inciting mechanisms of I/R injury is caused by an excessive generation of reactive oxygen species (ROS) and free radicals, which results in oxidative stress.[11, 12] The primary innate

defense mechanism against oxidative stress is the ROS scavenging effect. However, excessive production of ROS cannot be counteracted by the action of innate antioxidants. The imbalance between the pro-oxidants and antioxidant capacities of the endogenous radical-scavenging systems can trigger inflammatory reactions, cellular membrane and DNA damage, protein structure alteration and function impairment, and cell apoptosis, further exacerbating organ injuries.[13] Thus, therapies aimed at reducing the excessive oxidative stress seem logical.

Based on its redox-potential and powerful antioxidant capacity, vitamin C has been suggested as the most important form of antioxidant. The effect of intravenous (IV) vitamin C on I/R injury has been investigated in preclinical studies regarding the heart[14], hepatobiliary function[15] and skeletal muscle.[16] However, the results to date suggest that the organ protective effect of conventional doses of vitamin C are controversial. Since it would be difficult to effectively counteract overwhelming ROS production, high-dose vitamin C therapy has been proposed.[10, 17] Moreover, previous research has indicated that vitamin C levels are depleted in critically ill patients, in patients recovering from surgery, and in the subset of patients that are heading towards multi-organ failure.[18, 19] In that context, high-dose vitamin C therapy has been studied in critically ill patients, such as those with sepsis, but the results are also debatable.[18, 20, 21]

Yet, the degree of oxidative stress and inflammation, and pathophysiologic mechanisms of AKI may differ substantially between sepsis and renal I/R injury.[22] Currently, there is no comprehensive evidence for the effectiveness of high-dose vitamin C in renal I/R injury, while it deserves a high-priority considering the high incidence of AKI after cardiac surgery and its relationship with adverse outcome. Furthermore, although there are no consistent findings on the harmful effects of high-dose vitamin C exposure, potential concerns have been raised about renal insufficiency, hemolysis, and hypoglycemia.[23-25] Therefore, preclinical validation of the reno-protective efficacy and safety of high dose vitamin C is needed.

Therefore, our hypothesis is that high-dose vitamin C will be able to scavenge the

overwhelming ROS formation and downstream inflammatory reactions related to renal I/R injury and mitigate the development of AKI. The primary aim of the study is to validate reno-protective effects of high-dose IV vitamin C administration against renal I/R injury and provide an experimental rationale for the appropriate timing of perioperative vitamin C administration (either pre-emptive or upon reperfusion) that maximizes its protective efficacy.

II. MATERIALS AND METHODS

2.1. Animal Preparation

All animal experiments were approved by the committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine (IACUC No. 2020-0192), and were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (USA).

In this study, male Sprague-Dawley rats (10 to 12 weeks old, 250 to 300 g) were purchased from Orient Bio.Inc. (Korea). The rats were anesthetized with Rompun (10 mg/kg, Bayer DVM, Shawnee Mission, Kan) and Zoletil 50 (30 mg/kg, Virbac, Seoul, Korea), placed in a supine position. The rats were intubated with 16-gauge catheter and mechanically ventilated (Harvard Apparatus 683, Holliston, MA, USA) at 30–35 cycles/min. Body temperature was continuously monitored and maintained at around 37°C using an electric heating pad throughout the experiment.

2.2. Experimental models and Study groups

Based on preliminary studies, we used a proven rat model of renal I/R injury that showed the most consistent renal tubular injury without causing mortality.[26] Briefly, the rats received laparotomy and acute renal I/R injury was induced by clamping both renal arteries for 45 minutes using non-traumatic microvascular clamps, followed by 24 hours of reperfusion. I/R was confirmed by visual inspection of the kidney.

Initially, to determine the optimal timing for the administration of vitamin C in

relation to renal function, a dose of 200 mg/kg — the median doses among the options of 100 mg/kg, 200 mg/kg, and 300 mg/kg. — was administered intravenously via the tail vein. This was done either 1 hour prior to inducing ischemia or at the time of reperfusion. The rats were randomly categorized into 4 groups; Group 1: sham (animals with surgical procedure without renal artery clamping) (n = 6), group 2: IRC (animals with clamping and reperfusion of bilateral renal arteries) (n = 9), group 3: pre-vitC (vitamin C 200 mg/kg administration 1 h before clamping renal arteries) (n = 9), group 4: post-vitC (vitamin C 200 mg/kg administration upon reperfusion) (n = 9). Sham and IRC groups received equivalent volume of normal saline via the tail vein.

After establishing the ideal timing for vitamin C administration, which turned out to be at reperfusion, two additional groups were assigned to test different dose at that point. Group 5: V100 (vitamin C 100 mg/kg administered upon reperfusion) (n = 9), group 6: V300 (vitamin C 300 mg/kg administered upon reperfusion) (n = 9). The V200 group utilized samples from the previous experimental step (group 4).

2.3. Blood Urea Nitrogen and Creatinine Analysis

Serum samples were collected 24 h after reperfusion and analyzed for blood urea nitrogen (BUN) and creatinine (Cr) levels using the picric acid and diacetyl monoxime methods, respectively.[27]

2.4. Preparation of Kidney Tissue

At the end of the 24 h reperfusion, kidney tissue was harvested from the left kidney and cut into longitudinal sections. One piece was snap-frozen and stored at -80 °C for immunoblot analysis. The remaining piece was fixed in 10% formaldehyde and embedded in paraffin for histopathology examination at room temperature.

2.5. Histopathology Examination

Paraffin-embedded kidney tissues for histopathological examination were cut into 5

μm-slices and stained with hematoxylin eosin. Kidney tissues were cross sectioned through the midpoint to measure histologic damage. The severity of tubular damage was observed using an optical microscope of x100 magnification. The degree of tubular damage was graded on a scale from 0 to 3 (0 = no tubular necrosis; 1 = focal area of tubular necrosis involving <25% of the kidney; 2 = tubular necrosis involving 25% to 50% of the kidney; 3 = tubular necrosis involving >50% of the kidney) by a renal pathologist who was blinded to the study groups.

2.6. Enzyme-linked immunosorbent assay

Myeloperoxidase (MPO) activity in serum, markers of oxidative stress, was quantified using rat ELISA kits (LS Bio, Seattle, WA, USA). Serum levels of high-mobility group box 1 protein (HMGB1), interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α were measured using commercial rat ELISA kits (R&D Systems, Minneapolis, Minn, USA). All of them were analyzed according to the manufacturer's instructions.

2.7. Immunoblot Analysis

After necessary treatments, same amounts of protein from each group were processed to immunoblot assay as described previously.[28] Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-thioredoxin-interacting protein (TXNIP) (Santa Cruz Biotechnology, Dallas, TX, USA), anti-NADPH oxidase 4 (NOX4), anti-NOD like receptor 3 (NLRP3), anti-apoptosis-associated speck-like protein (ASC), anti-caspase-1, and anti-interleukin (IL)-1β (Cell Signaling Technology, Beverly, Mass). And anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technology, Beverly, Mass) was used as a loading control. Protein expression was measured by densitometry and normalized by GAPDH. Each experiment was performed in triplicate.

2.8. Statistical Analysis

The data are expressed as mean ± standard deviation. Multiple comparisons among groups were analyzed by Student's t-test and 1-way analysis of variance (ANOVA) followed by Bonferroni correction or repeated measures ANOVA. *P*-value <0.05 was considered statistically significant.

III. RESULTS

3.1. Optimal timing and dosage of vitamin C treatment on I/R induced renal dysfunction

Firstly, the optimal timing of vitamin C administration was assessed using the median dose (200 mg/kg). Renal I/R injury resulted in significant increase in BUN and Cr, and the magnitude of attenuating their increase was significantly greater when vitamin C was administered upon reperfusion compared to pre-ischemia. Moreover, pre-ischemia vitamin C administration did not result in significant attenuation of I/R-induced increase in BUN. Secondly, among the various doses of vitamin C treatment, serum BUN and creatinine levels significantly decreased in the V100 and V200 groups compared to the IRC group (*P* <0.05). This reduction was more pronounced in the V200 group, while a higher dose (V300) could not significantly attenuate the increase in BUN and Cr when compared to the IRC group (Fig. 1).

3.2. The effect of vitamin C treatment on I/R induced renal tubular damage

Renal I/R injury in rats resulted in a significant increase in the number of tubular cell deaths and the degree of renal tubular damage, in comparison with the sham group. The degree of renal tubular damage in the group that received vitamin C before ischemia were comparable to that of the IRC group. In contrast, the group treated with vitamin C during reperfusion displayed a significant reduction in tubular damage similar to that of the sham group, and a pronounced beneficial effect compared to the pre-ischemia vitamin C treatment group. Therefore, subsequent experiments were focused solely on post-

reperfusion therapy.

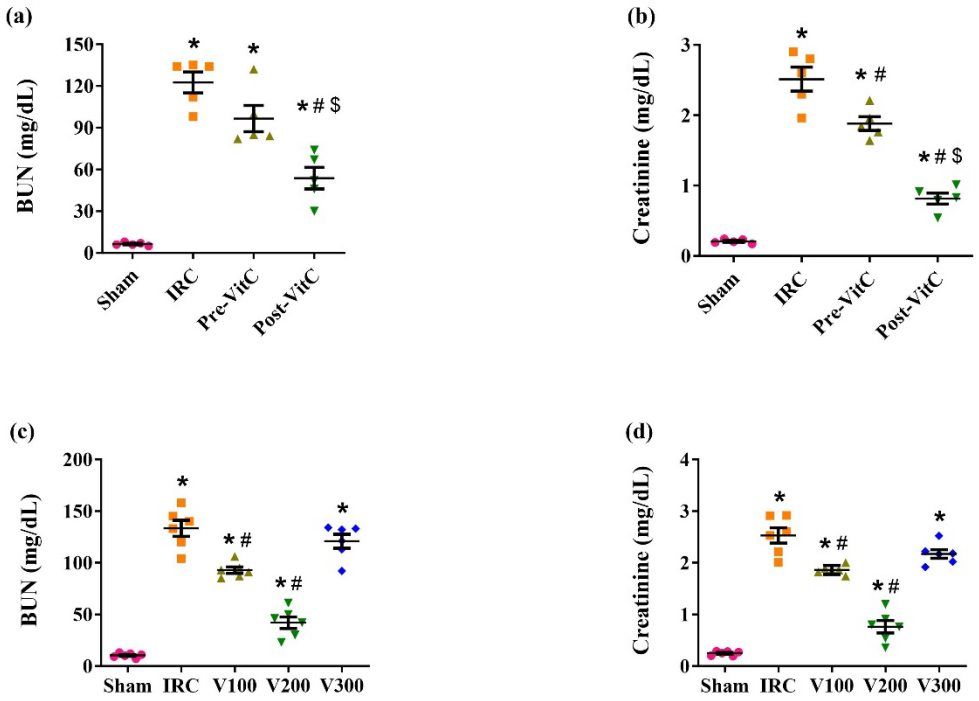

Figure 1. Effects of vitamin C treatment on renal function. Renal ischemia-reperfusion (I/R) injury produced a significant increase in serum blood urea nitrogen (BUN) (a), Creatinine levels (b), these increases were significantly mitigated in the group treated with vitamin C during reperfusion (post-vitC group). The administration of 100 mg/kg and 200 mg/kg doses of vitamin C upon reperfusion resulted in a substantial reduction in serum BUN and creatinine levels (c), (d), as compared with the ischemia-reperfusion control (IRC) group. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) and reperfusion (24 h); pre-vitC = vitamin C 200 mg/kg administered 1 h before ischemia; post-vitC = vitamin C 200 mg/kg administered upon reperfusion; V100, V200, V300 = respective doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg of vitamin C, administered upon reperfusion. * P <0.05 versus sham, # P <0.05 versus IRC, $ P <0.05 versus Pre-vitC.

The V100 group showed a reduction in tubular damage, though it was not statistically

significant when compared to either the sham or the IRC groups. The V200 group demonstrated notable decrease in the degree of tubular damage. In contrast, the V300 group did not exhibit any significant protective effect when compared to the IRC group (Fig. 2).

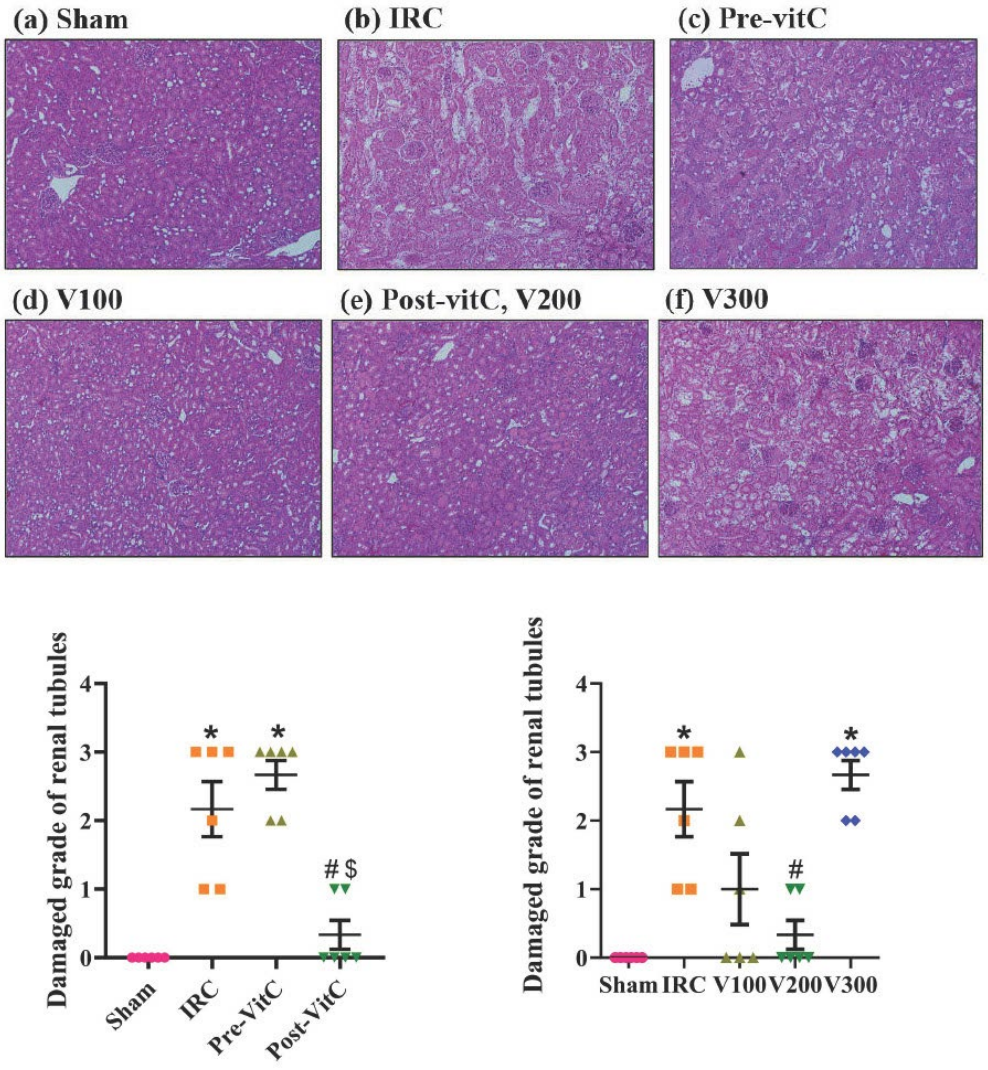

Figure 2. Effects of vitamin C treatment during reperfusion on histology of renal tubules. The degree of tubular damage assessed using a scale of 0 to 3 (0 = no tubular necrosis, 1 = focal area of tubular necrosis involving <25% of the kidney, 2 = tubular necrosis involving

25% to 50% of the kidney, 3 = tubular necrosis involving >50% of the kidney) at 24 hours after ischemia and reperfusion. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) and reperfusion (24 h); pre-vitC = vitamin C 200 mg/kg administered 1 h before ischemia; post-vitC = vitamin C 200 mg/kg administered upon reperfusion; V100, V200, V300 = respective doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg of vitamin C, administered upon reperfusion. * P <0.05 versus sham, # P <0.05 versus IRC, $ P <0.05 versus Pre-vitC.

3.3. The effect of post-reperfusion Vitamin C treatment on oxidative stress and reactive oxygen species formation

There were significant increases in serum MPO activity in I/R injured groups compared with the sham group reflecting the development of significant oxidative stress. Compared to the IRC group, the activity of MPO was significantly decreased in the V200 group, which was comparable to the sham group.

Similarly, TXNIP and NOX4 levels of the renal tissue, which are well-known mediators of ROS formation, were significantly increased in the IRC group compared with the sham group. This increase in TXNIP level could be significantly attenuated in the V100 and V200 groups, whereas it was even more increased in the V300 group compared with the IRC group. In contrast, the I/R-induced increase in NOX4 level could be significantly attenuated in all vitamin C treatment groups while the decrease was most prominent in the V100 group almost approaching the corresponding level of the sham group (Fig. 3).

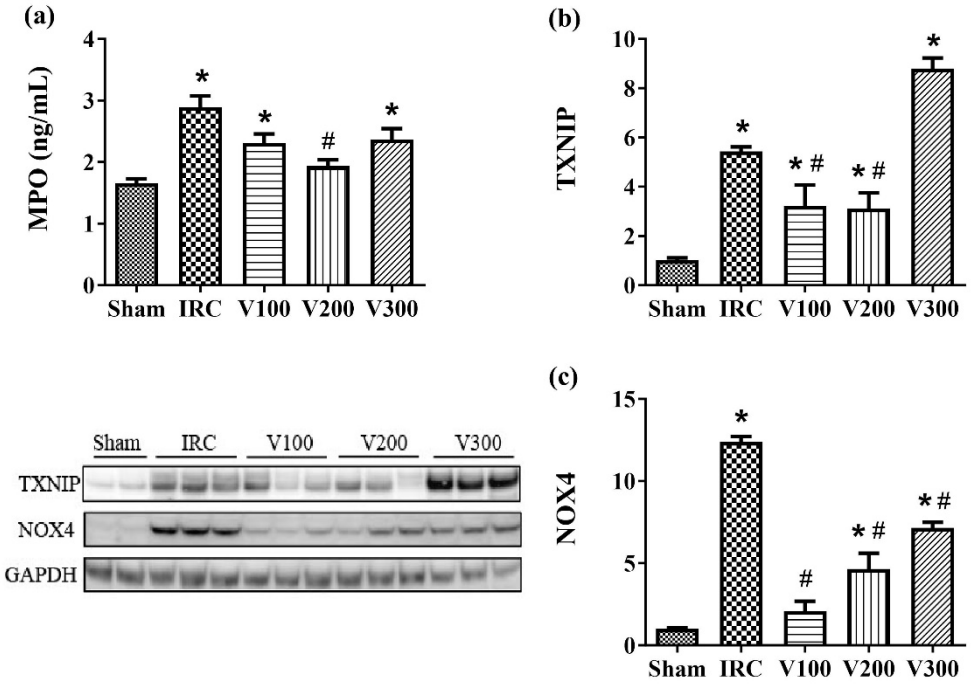

Figure 3. Effects of vitamin C treatment during reperfusion on oxidative stress markers. (a) Myeloperoxidase (MPO) levels in the serum, as assessed by an Enzyme-Linked Immunosorbent Assay (ELISA). Protein levels of (b) Thioredoxin Interacting Protein (TXNIP) and (c) NADPH Oxidase 4 (NOX 4) in kidney tissues were evaluated through an immunoblot assay. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) and reperfusion (24 h); V100, V200, V300 = respective doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg of vitamin C, administered upon reperfusion. * P <0.05 versus sham, # P <0.05 versus IRC.

3.4. The effect of post-reperfusion vitamin C treatment on serum inflammatory markers

I/R resulted in significant increases in serum concentrations of HMGB1, TNF-α, IL-

1β and IL-6 (P <0.05). I/R-induced increase in HMGB1 levels could only be significantly attenuated in the V100 and V200 groups, which were all still significantly higher than that of the sham group. Similar results could be observed in TNF-α levels, while the decrease was most prominent in the V200 group, which showed no statistically significant difference compared with the sham group. When compared with the IRC group, IL-1β levels were significantly decreased only in the V100 and V200 groups and not in the V300 group. Moreover, the degree of this decrease approached the level of the sham group. In the case of IL-6 levels, all of the V100, V200, and V300 groups showed a significant decrease compared to the IRC group, and the levels of the V100 and V200 groups were comparable to that of the sham group (Fig. 4).

3.5. The effect of post-reperfusion vitamin C treatment on inflammasome of the renal tissue

The levels of NLRP3, ASC, CASPASE-1 and IL-1β in the kidney were all significantly increased after I/R injury. The increase in NLRP3 and ASC levels could be significantly attenuated in all vitamin C-treated groups similar to that of the corresponding sham group. Caspase-1 and IL-1β levels could only be significantly decreased in the V100 and V200 groups compared to the IRC group, while they were all significantly higher than that of the corresponding sham group (Fig. 5).

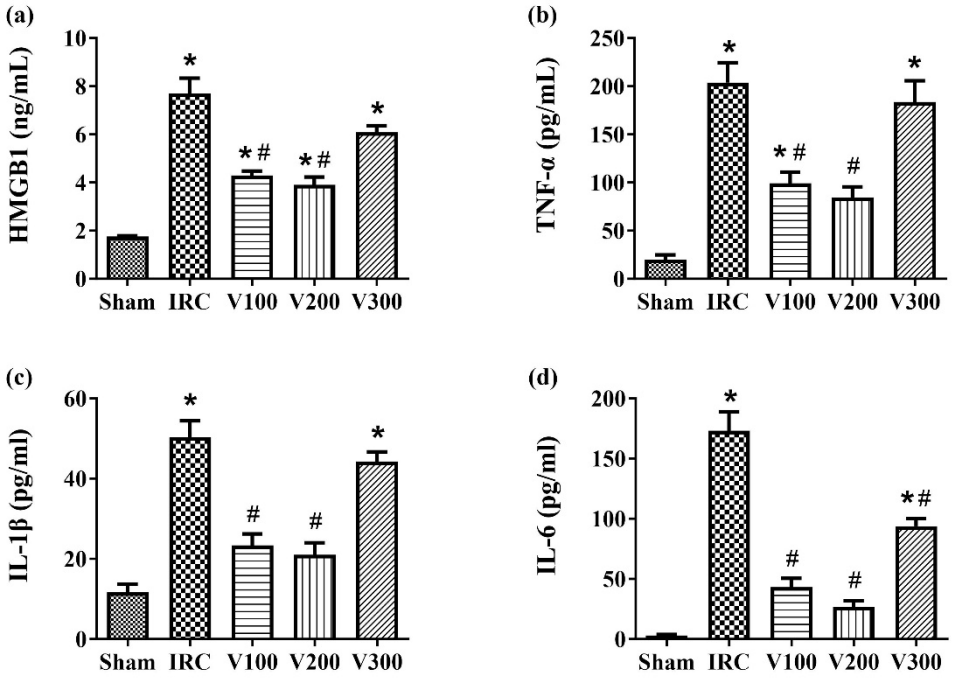

Figure 4. Effects of vitamin C treatment during reperfusion on inflammatory markers. The protein levels of (a) High Mobility Group Box 1 (HMGB1), (b) Tumor Necrosis Factor Alpha (TNF-α), (c) Interleukin-1 Beta (IL-1β), and (d) Interleukin-6 (IL-6) in the serum are assessed by an Enzyme-Linked Immunosorbent Assay (ELISA). Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) and reperfusion (24 h); V100, V200, V300 = respective doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg of vitamin C, administered upon reperfusion. * P <0.05 versus sham, # P <0.05 versus IRC.

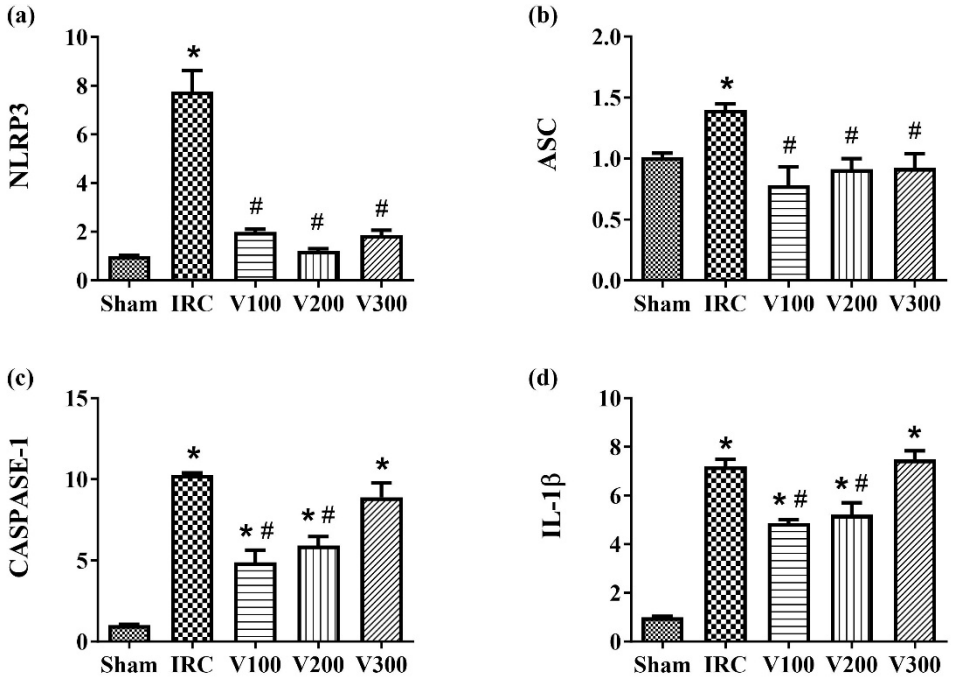

Figure 5. Effects of vitamin C treatment during reperfusion on inflammasome components. The protein levels of (a) NLR Family Pyrin Domain Containing 3 (NLRP3), (b) Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC), (c) Caspase-1, and (d) Interleukin-1 Beta (IL-1β) in kidney tissues are analyzed through an immunoblot assay. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) and reperfusion (24 h); V100, V200, V300 = respective doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg of vitamin C, administered upon reperfusion. * P <0.05 versus sham, # P <0.05 versus IRC.

IV. DISCUSSION

In the current study, we demonstrated that high-dose vitamin C administration upon reperfusion exhibited a significant reno-protective effect in a rat model of renal I/R injury, which was significantly more effective than pre-ischemic administration. This beneficial effect was associated with attenuation of oxidative stress-related major regulators such as TXNIP and NOX4, as well as mitigation of subsequent ROS-linked NLRP3 inflammasome

and release of HMGB1, which are foremost primary triggers of inflammatory response that further promote renal injury. The most effective dose of vitamin C was found to be 200 mg/kg in terms of mitigating the aforementioned I/R-induced oxidative stress and inflammation, and ultimately renal dysfunction and renal tubular damage.

ROS plays an important role in innate defense mechanisms. However, uncontrolled or excessive ROS generation can lead to oxidative stress and tissue damage, contributing to the development of AKI after I/R.[11] During the inevitable period of reperfusion of major organs, excessive production of ROS becomes a key trigger that activates inflammatory response of various pathways, including NLRP3 inflammasome by way of TXNIP and NOX4, which is a major trigger of downstream inflammatory response.[29-31] Also, renal I/R injury has been shown to promote increased HMGB1 release, which acts as a key inciting substance linking the initial injury from ischemic energy depletion and the following inflammatory response that incurs further cell death.[32, 33] Accordingly, inhibition of the foremost inciting events that aggravate organ damage after I/R injury, oxidative stress and inflammation, would serve as a useful strategy to protect major organs from ischemia-reperfusion injury. In that context, we have previously investigated the reno-protective role of propofol[26], dexmedetomidine[34], and ethyl pyruvate[35] in a rat model of renal I/R injury. These substances contribute to reno-protection by exhibiting antioxidant or anti-inflammatory properties.

In line with these findings, vitamin C, acting as a potent antioxidant, safeguards cells from damage caused by ROS and free radicals, volatile compounds known to instigate tissue damage and inflammation.[36] Unlike most animals, humans are incapable of endogenous vitamin C production due to the lack of the enzyme L-gulonolactone oxidase. Consequently, humans must depend on dietary intake to meet their daily vitamin C requirements.[37] However, under certain circumstances, such as in critically ill patients, post-surgery patients, or those approaching multi-organ failure, where vitamin C levels are often diminished, higher doses of vitamin C may be necessary.[17, 18] Recently, there has been ongoing interest in the clinical application of high-dose vitamin C in critically ill patients

with viral infections, including COVID-19. While some studies have shown positive effects, the evidence remains controversial.

Research into the protective effects of high-dose vitamin C on various organs under I/R injury and septic shock conditions has shown promising, yet inconsistent results.[18, 20, 21] Although potentially beneficial, use of high-dose vitamin C carries some concerns and must be employed with caution. High doses may lead to acute kidney injury from the metabolite oxalic acid, acute hemolysis, particularly in individuals with glucose-6-phosphate dehydrogenase deficiency, and hypoglycemia due to glucose meter errors.[20, 23-25] Notably, a recent study exploring early high-dose vitamin C administration in patients with septic shock reported an increase in organ failure and mortality rates[20], contradicting previous findings. Nevertheless, given that conventional doses are insufficient to counteract the excessive oxidative stress that occurs during I/R injury, we hypothesized that high-dose vitamin C may provide reno-protective benefits during such injury.

Our results demonstrated that administering vitamin C upon reperfusion rather than prior to ischemia, not only led to a significant decrease in BUN and Cr levels but also exhibited a more beneficial effect on renal tissue protection, as evidenced by renal histology. This suggests a protective role against renal dysfunction. This finding regarding the administration timing is especially valuable in the surgical setting as it would theoretically not hinder the anesthetic pre-conditioning effect. Inhalation anesthetics are well-known for their conditioning effect on major organs, which ameliorates I/R injury. However, anesthetic pre-conditioning requires the production of ROS to trigger downstream protective pathways.[38, 39] Therefore, there has been concerns that the use of antioxidants such as vitamin C could negate the beneficial conditioning effects of anesthetics by neutralizing the necessary redox signals (ROS generation). Importantly, our study revealed that administering vitamin C during reperfusion provided greater kidney protection than when it was given preemptively. A combination of anesthetic pre-conditioning and vitamin C post-treatment may have a synergistic effect, protecting the kidneys from I/R injury. Moreover, intravenous administration bypasses the absorption step, allowing plasma

concentrations to increase rapidly and resulting in high bioavailability. The antioxidant action of intravenously administered vitamin C begins immediately after administration and continues depending on the effective concentration in the body.[17] Since ROS production is maximized during reperfusion, administering vitamin C during this phase could be more advantageous than administering it before ischemia, and our results support this.

Interestingly, the reno-protective effect did not follow a dose-dependent pattern either in terms of serum BUN, Cr levels or histology. The groups receiving 100 mg/kg and 200 mg/kg of vitamin C displayed significant reductions in BUN and Cr levels and demonstrated histological evidence of protection against kidney injury. Yet, this protective response could not be escalated in the group given a 300 mg/kg dosage of vitamin C, which was similar to the IRC group both in terms of serum BUN and Cr levels and histological evaluations, thereby suggesting a potentially diminished efficacy beyond a certain dosage. Vitamin C primarily acts as an antioxidant. However, some in vitro studies using it as an adjuvant treatment for cancer patients have shown that it acts as a prooxidant by producing extracellular hydrogen peroxide (H_2O_2), which is cytotoxic to cancer cells at above physiological concentrations.[40, 41] Current data suggests that H_2O_2 generated by high-dose vitamin C use is harmless to normal cells[42], but the underlying mechanism remains ambiguous. Moreover, the effects on I/R-injured cells are yet to be determined. Furthermore, it may be prudent to examine whether there's a ceiling dose for kidney protection associated with H_2O_2 production from elevated doses of vitamin C. Additionally, it is worthy to investigate whether these observations are associated with an increased incidence of oxalate nephropathy, a type of acute kidney injury that can occur due to elevated urinary oxalate excretion resulting from prolonged high-dose vitamin C use, which could not be observed in the current study employing a single dose. Further studies are warranted to discern if surpassing therapeutic doses could diminish renal protection or even promote renal damage.

Furthermore, the main objective of the study on the optimal dose of vitamin C was

to assess critical mediator levels to discern if they could effectively restrain excessive ROS production, alleviate the activation of downstream inflammasomes linked to ROS, and sufficiently control the inflammatory response which is crucial to I/R injury and organ damage.

MPO, an enzyme primarily found in neutrophils, contributes to the immune response by generating ROS to kill invading microbes. Its increased activity during reperfusion instigates ROS production, promotes inflammation, and facilitates polymorphonuclear leukocyte infiltration into tissues. Many previous studies addressing I/R injury have used MPO as a biochemical marker after I/R in vivo.[43] In this study, 200 mg/kg vitamin C administration upon reperfusion effectively decreased MPO activity (similar to the level of the sham group) prompted by renal I/R injury. We can surmise that administering vitamin C 200mg/kg during reperfusion could potentially have a protective role by reducing ROS production.

The NLRP3 inflammasome is a critical mediator of oxidative stress and the subsequent inflammatory responses. Under various stress conditions, such as those created by an excess of ROS, NLRP3 activation can occur. Several studies emphasized its role in ischemic AKI and suggests NLRP3 knock-out mice are protected against ischemic AKI, highlighting the mROS-TXNIP-NLRP3 pathway as a significant mechanism of ischemic AKI.[29-31] Our results showed that vitamin C administration substantially reduced the oxidative stress markers TXNIP and NOX4 and hindered the activation of the NLRP3 inflammasome, indicating an efficient suppression of the complex interplay between oxidative stress and subsequent inflammation. Therefore, taking into account the decrease in MPO activity along with the reduction in TXNIP and NOX levels, it could be suggested that administering vitamin C at the time of reperfusion might be advantageous in mitigating oxidative stress, primarily by inhibiting the production of ROS. The dose demonstrating the highest efficacy across all these processes was 200 mg/kg of vitamin C.

High-mobility group box 1 (HMGB1) is an endogenous molecule that has been identified as a vital inflammatory mediator in previous studies, rising under various stress

conditions. Notably, HMGB1 is a significant inflammatory factor primarily released by ischemic injury, though it can also be activated by ROS through a different pathway than NLRP3.[32, 33] Experimental studies suggest that antioxidants like ethyl pyruvate can shield organs from I/R injury by reducing HMGB1 release.[35] In this scenario, we evaluated whether vitamin C, an antioxidant, could mitigate HMGB1 activation. The findings revealed that 100 mg/kg and 200 mg/kg of vitamin C, administered during reperfusion, significantly curtailed the surge in HMGB1 triggered by I/R injury. Both NLRP3 and HMGB1 were suppressed, thereby diminishing inflammatory markers such as IL-1β, IL-6, and TNF-α. These beneficial outcomes are thought to protect against renal I/R injury.

In our research, we administered 100 mg, 200 mg, and 300 mg/kg dosages of vitamin C intravenously to rats. Recent studies suggest a minimum of 2 g/day of intravenous vitamin C for critically ill patients to achieve stable plasma levels.[18] Considering the surge of ROS during I/R injury, our hypothesis was that the required vitamin C concentrations to maintain normal plasma levels would align with those prescribed for critically ill patients. Utilizing the Human Equivalent Dose (HED) calculation[44, 45], and considering a Km factor of 12 for our experimental rats, which weighed approximately 300 g, and a Km factor of 37 for a 60 kg adult human, we determined that the human dose of 2 g/day is roughly equivalent to a rat dose of 100 mg/kg. Additionally, some studies indicated that vitamin C dosages as high as 6 g/day were not contraindicated even for patients with G6PD deficiency[46], who are most vulnerable to potential harms. Hence, prioritizing the study's safety, the peak dosage was aligned with an equivalent of 6 g/day for humans. Following this rationale, the dose corresponding to a 6 g daily intake for a 60 kg human would approximate to 300 mg/kg for a 300 g rat. In this study, we sought to determine the effectiveness and safety of high doses of vitamin C, beyond typical physiological amounts, to protect the kidneys from I/R injury. Therefore, we focused on doses that reflected HED values between approximately 2 g and 6 g per day.

Overall, the strength of this study is that it provides primary evidence of the reno-protective properties of high-dose vitamin C against I/R injury and its timing of

administration and dosage. Given its safety, cost-effectiveness and accessibility, we believe that vitamin C could be used pre-emptively to prevent AKI, a common complication following cardiac surgery that involves an inevitable period of ischemia and reperfusion and systemic inflammation.

The limitations of this study are as follows. First, due to unique physiological and metabolic disparities, such as the ability to synthesize vitamin C, it is not always possible to directly translate the results from these studies to humans. Second, we successfully established a rat model of renal ischemic reperfusion injury by performing a laparotomy and clamping and releasing the femoral artery. However, caution must be exercised when applying these results to human surgical situations. The specific systemic physiological responses during cardiopulmonary bypass, and the actual human surgical environment encountered in human cardiac surgeries may not be completely replicated in rat models. This could potentially restrict the range and practicality of our research conclusions. Lastly, our study didn't assess the risk of hemolysis or hypoglycemia at the given doses, which poses another limitation. To ascertain its safety, more investigations are needed that consider different methods of administration, dosage levels, and disease states.

V. CONCLUSION

This study showed that high dose of vitamin C (200 mg/kg) administration upon reperfusion conveyed reno-protective effects against renal I/R injury via reduction of oxidative stress and inflammation. These beneficial influences were associated with a decrease in MPO activity and a reduction in the oxidative stress markers TXNIP and NOX4. These findings suggest that high-dose vitamin C can effectively attenuate the overwhelming oxidative stress. Furthermore, there was a reduction in the levels of HMGB1 and NLRP3, which are closely linked to ischemia (energy deprivation) and reperfusion (ROS generation) injury, respectively, the most crucial triggers of downstream inflammatory response following I/R injury that promote further cell deaths. These findings provide experimental evidence for the use of high dose vitamin C with antioxidant and anti-

inflammatory effects in preventing kidney injury associated with major surgeries that inevitably accompany various degrees of I/R injury as well as inflammation.

REFERENCES

1. Vaage J, Valen G. Pathophysiology and mediators of ischemia-reperfusion injury with special reference to cardiac surgery. A review. Scand J Thorac Cardiovasc Surg Suppl 1993;41:1-18.
2. Levy JH, Tanaka KA. Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 2003;75(2):S715-S720.
3. Hill A, Clasen KC, Wendt S, Majoros ÁG, Stoppe C, Adhikari NKJ, et al., Effects of Vitamin C on Organ Function in Cardiac Surgery Patients: A Systematic Review and Meta-Analysis. Nutrients 2019;11(9):2103.
4. Rosner MH, Portilla D, Okusa MD. Cardiac surgery as a cause of acute kidney injury: pathogenesis and potential therapies. J Intensive Care Med 2008;23(1):3-18.
5. Loef BG, Epema AH, Smilde TD, Henning RH, Ebels T, Navis G, et al. Immediate postoperative renal function deterioration in cardiac surgical patients predicts in-hospital mortality and long-term survival. J Am Soc Nephrol 2005;16(1):195-200.
6. Lassnigg A, Schmidlin D, Mouhieddine M, Bachmann LM, Druml W, Bauer P, et al., Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: a prospective cohort study. J Am Soc Nephrol 2004;15(6):1597-1605.
7. Cho JS, Shim JK, Lee S, Song JW, Choi N, Lee S, et al. Chronic progression of cardiac surgery associated acute kidney injury: Intermediary role of acute kidney disease. J Thorac Cardiovasc Surg 2021;161(2):681-8.e3.
8. Bain CR, Myles PS, Corcoran T, Dieleman JM. Postoperative systemic inflammatory dysregulation and corticosteroids: a narrative review. Anaesthesia 2023;78(3):356-70.
9. Dvirnik N, Belley-Cote EP, Hanif H, Devereaux PJ, Lamy A, Dieleman JM, et al. Steroids in cardiac surgery: a systematic review and meta-analysis. Br J Anaesth 2018;120(4):657-67.

10. Laffey JG, Boylan JF, Cheng DC. The systemic inflammatory response to cardiac surgery: implications for the anesthesiologist. Anesthesiology 2002;97(1):215-52.
11. Malek M, Nematbakhsh M. Renal ischemia/reperfusion injury; from pathophysiology to treatment. J Renal Inj Prev 2015;4(2):20-7.
12. Baikoussis NG, Papakonstantinou NA, Verra C, Kakouris G, Chounti M, Hountis P, et al. Mechanisms of oxidative stress and myocardial protection during open-heart surgery. Ann Card Anaesth 2015;18(4):555-64.
13. Bandyopadhyay U, Das D, Banerjee RK. Reactive oxygen species: oxidative damage and pathogenesis. Current science, 1999: p. 658-66.
14. Rodrigo R, Prieto JC, Castillo R. Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical applications. Clin Sci (Lond) 2013;124(1):1-15.
15. Seo MY, Lee SM. Protective effect of low dose of ascorbic acid on hepatobiliary function in hepatic ischemia/reperfusion in rats. J Hepatol 2002;36(1):72-7.
16. Saçar M, Özcan V, Aybek H, Önem G, Demir S, Gökşin İ, et al. Vitamin C and iloprost attenuate skeletal muscle injury caused by ischemia-reperfusion of the lower extremities. Turkish J Thorac Cardiovasc Surg 2005;13(4):374-8.
17. Spoelstra-de Man AME, Elbers PWG, Oudemans-van Straaten HM. Making sense of early high-dose intravenous vitamin C in ischemia/reperfusion injury. Crit Care 2018;22(1):70.
18. de Grooth HJ, Manubulu-Choo WP, Zandvliet AS, Spoelstra-de Man AME, Girbes AR, Swart EL, et al. Vitamin C Pharmacokinetics in Critically Ill Patients: A Randomized Trial of Four IV Regimens. Chest 2018;153(6):1368-77.
19. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, et al. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. Crit Care Med 1996;24(3):392-7.
20. Lamontagne F, Masse MH, Menard J, Sprague S, Pinto R, Heyland DK, et al.

Intravenous Vitamin C in Adults with Sepsis in the Intensive Care Unit. N Engl J Med 2022;386(25):2387-98.

21. Zabet MH, Mohammadi M, Ramezani M, Khalili H. Effect of high-dose Ascorbic acid on vasopressor's requirement in septic shock. J Res Pharm Pract 2016;5(2):94-100.
22. Schrier RW, Wang W. Acute renal failure and sepsis. N Engl J Med 2004;351(2):159-69.
23. Cossey LN, Rahim F, Larsen CP. Oxalate nephropathy and intravenous vitamin C. Am J Kidney Dis 2013;61(6):1032-5.
24. Quinn J, Gerber B, Fouche R, Kenyon K, Blom Z, Muthukanagaraj P. Effect of High-Dose Vitamin C Infusion in a Glucose-6-Phosphate Dehydrogenase-Deficient Patient. Case Rep Med 2017;2017:5202606.
25. Sartor Z, Kesey J, Dissanaike S. The effects of intravenous vitamin C on point-of-care glucose monitoring. J Burn Care Res 2015;36(1):50-6.
26. Yoo YC, Yoo KJ, Lim BJ, Jun JH, Shim JK, Kwak YL. Propofol attenuates renal ischemia-reperfusion injury aggravated by hyperglycemia. J Surg Res 2013;183(2):783-91.
27. Wybenga DR, Di Giorgio J, Pileggi VJ. Manual and automated methods for urea nitrogen measurement in whole serum. Clin Chem 1971;17(9):891-5.
28. Jun JH, Shim JK, Ryoo HM, Kwak YL. Erythropoietin-activated ERK/MAP kinase enhances GATA-4 acetylation via phosphorylation of serine 261 of GATA-4. J Cell Physiol 2013;228(1):190-7.
29. Wen Y, Liu YR, Tang TT, Pan MM, Xu SC, Ma KL, et al. mROS-TXNIP axis activates NLRP3 inflammasome to mediate renal injury during ischemic AKI. Int J Biochem Cell Biol 2018;98:43-53.
30. Kim HJ, Lee DW, Ravichandran K, O Keys D, Akcay A, Nguyen Q, et al., NLRP3 inflammasome knockout mice are protected against ischemic but not cisplatin-induced acute kidney injury. J Pharmacol Exp Ther 2013;346(3):465-72.

31. Bakker PJ, Butter LM, Claessen N, Teske GJ, Sutterwala FS, Florquin S, et al. A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion. Am J Pathol 2014;184(7):2013-22.
32. Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-mobility group box 1, oxidative stress, and disease. Antioxid Redox Signal 2011;14(7):1315-35.
33. Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U, Wyburn KR, et al. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol. 2010;21(11):1878-90.
34. Kim SH, Jun JH, Oh JE, Shin EJ, Oh YJ, Choi YS. Renoprotective effects of dexmedetomidine against ischemia-reperfusion injury in streptozotocin-induced diabetic rats. PLoS One 2018;13(8):e0198307.
35. Jun JH, Song JW, Shin EJ, Kwak YL, Choi N, Shim JK. Ethyl pyruvate is renoprotective against ischemia-reperfusion injury under hyperglycemia. J Thorac Cardiovasc Surg 2018;155(4):1650-1658.
36. Levine M. New concepts in the biology and biochemistry of ascorbic acid. N Engl J Med 1986;314(14):892-902.
37. Naidu KA. Vitamin C in human health and disease is still a mystery? An overview. Nutr J 2003;2:7.
38. Zhao J, Wang F, Zhang Y, Jiao L, Lau WB, Wang L, et al. Sevoflurane preconditioning attenuates myocardial ischemia/reperfusion injury via caveolin-3-dependent cyclooxygenase-2 inhibition. Circulation 2013;128(11 Suppl 1):S121-S129.
39. Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC. Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. Br J Anaesth 2003;91(4):551-565.
40. Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC, et al. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci USA

2008;105(32):11105-9.

41. Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, et al. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci USA 2005;102(38):13604-9.
42. Padayatty SJ, Levine M. Vitamin C: the known and the unknown and Goldilocks. Oral Dis 2016;22(6):463-93.
43. Laight DW, Lad N, Woodward B, Waterfall JF. Assessment of myeloperoxidase activity in renal tissue after ischemia/reperfusion. Eur J Pharmacol 1994;292(1):81-8.
44. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7(2):27-31.
45. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22(3):659-61.
46. Honore PM, Spapen HD, Marik P, Boer W, Oudemans-van Straaten H. Dosing vitamin C in critically ill patients with special attention to renal replacement therapy: a narrative review. Ann Intensive Care 2020;10(1):23.

ABSTRACT (IN KOREAN)

고용량 비타민 C가 신장의 허혈-재관류 손상에 미치는 영향

<지도교수 심재광>

연세대학교 대학원 의학과

고서희

배경

급성신부전은 환자 예후에 악영향을 미치며, 심장 수술 후 흔하게 발생하는 합병증이다. 급성신부전 발생의 주요한 원인은 신장의 허혈-재관류 손상이며, 이는 수술 스트레스와 심폐우회술과 관련된 전신적인 염증 반응과도 관련이 있다. 급성신부전 발생을 줄이기 위해 염증 반응을 억제하고 허혈-재관류 손상을 완화하기 위한 다양한 실험적 방법이 시도되었으나, 임상에서는 유효하지 않았다. 비타민 C는 체내에서 산화 스트레스에 대응하는 일차 방어 기전으로, 허혈-재관류 손상에 대한 잠재적 치료제로 제안되었다. 그러므로, 고용량의 비타민 C를 투여하면 심장 수술로 인해 유발되는 과도한 산화 스트레스를 완화하여 급성신부전을 발생시키는 복잡한 병태생리학적 기전을 억제하여 급성신부전 발생을 줄일 수 있을 것으로 보인다. 이에 고용량 비타민 C 투여의 신장의 허혈-재관류 손상에 대한 보호 효과에 관하여 연구하고자 하였다.

방법

실험은 크게 두 단계로 수행되었다. 먼저 최적의 투여 시점을 결정한 다음 최적의 용량을 결정하였다. Sprague-Dawley 쥐를 대상으로 하여, 무작위로 sham, IRC (허혈-재관류 시점에 식염수 투여), pre-vitC (비타민 C 전 투여 후 허혈-재관류), post-vitC (허혈-재관류 시점에 비타민C 투여)의 4개의 군으로 배정하였다. 신장의 허혈-재관류는 45분간의 허혈(신장 동맥 결찰)과 24시간의 재관류로 유도하였다. 비타민 C 200 mg/kg는 허혈 1시간 전에 (pre-vitC) 혹은 재관류 시점에 (post-vitC) 정맥 주입되었다. 재관류 후 신장 조직과 신기능을

평가하였다. 이전 단계에서 확인된 최적의 투여 시기 (재관류 시점)를 사용하여 다양한 용량에서 평가하기 위해 V100 (비타민 C 100mg/kg), V300 (비타민 C 300mg/kg) 두 개의 그룹을 더 배정하였다. V200 그룹은 이전 단계 실험의 샘플을 사용하였다.

결과
재관류 시점에 투여한 비타민 C는 신장 허혈-재관류 손상 후 신장 기능 장애와 신장 조직의 구조 손상을 개선했다. 재관류 중 투여한 100 mg/kg 및 200 mg/kg의 비타민 C는 산화 스트레스 마커인 골수세포형 과산화효소 (Myeloperoxidase, MPO), 티오레독신 결합단백질(Thioredoxin-interacting protein, TXNIP), 및 NADPH 산화효소 4 (NOX4)를 감소시켰다. 또한, 비타민 C는 sham 그룹과 비교하여 HMGB1, IL-1β, IL-6, 및 TNF-α 발현을 감소시켜 ROS 관련 염증 반응을 완화하였다. 재관류 시점에 투여한 비타민 C 100mg/kg 및 200mg/kg는 허혈-재관류로 인해 유발되는 NLRP3 인플라마좀 (inflammasome)의 구성인자들을 효과적으로 억제하였다.

결론
재관류 시점에 투여한 200 mg/kg 용량의 고용량 비타민 C는 산화 스트레스를 효과적으로 감소시킴으로써 신장의 허혈-재관류 손상을 완화할 수 있었다. 이 효과는 HMGB1 방출 감소와 NLRP3 인플라마좀 활성 억제, 그리고 이와 관련된 후속 염증 반응의 감소와 관련되어 있었다.

핵심 되는 말: 비타민 C, 아스코르빈산, 급성신부전, 허혈-재관류 손상

PUBLICATION LIST

1. Ko SH, Song JW, Shim JK, Soh S, Kwak YL. Low Intraoperative Cerebral Oxygen Saturation Is Associated with Acute Kidney Injury after Off-Pump Coronary Artery Bypass. J Clin Med 2023;12(1):359.