Effect of Vitamin C (Ascorbic Acid) as an Antioxidant in Reducing Cellular Injury Following Renal Reperfusion in Wistar Rats

Nithyananda Aanantharya Vinodini,1 Yogesh Tripathi,2 Coimbatore Vasudevan Raghuveer,3 Kamath Asha4

Background: Renal ischemia-reperfusion (I/R) injury occurs as a result of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS cause oxidative stress, which results in an imbalance between oxidants such as ROS and antioxidants. The objective of this study was to evaluate the protective effect of the antioxidant vitamin C compared to the effect of vitamin E on renal I/R.

Methods: Wistar albino rats were divided into six groups. There were three control groups: Group 1—normal control; Group 2—sham control; Group 3—untreated experimental control. There were three experimental groups where rats were pretreated with a vitamin for 30 days: Group 4—pretreated with vitamin E; Group 5—pretreated with vitamin C; Group 6—pretreated with a combination of vitamins E and C. On day 31, all groups except normal and sham control underwent 60 minutes of renal ischemia followed by reperfusion for 10 minutes. After this, the kidney was removed and homogenized. The homogenate was used for the biochemical estimations of lipid peroxidation, glutathione (GSH) and superoxide dismutase (SOD).

Results: Ischemia followed by reperfusion led to a significant increase in tissue lipid peroxidation and a decrease in GSH and SOD levels (Group 3). However, in Groups 4, 5 and 6, where the rats were pretreated with vitamin C or E or a combination of both, there was a decrease in lipid peroxidation, and an increase in GSH and SOD levels. Though a decrease in lipid peroxidation was observed in all three vitamin-pretreated groups, it was not as low as in the normal control group. There were no statistically significant differences among the three vitamin-pretreated groups.

Conclusion: Both antioxidants, singly and in combination, showed equal beneficial effects in reducing renal injury. [Hong Kong J Nephrol 2009;11(1):9–13]

Key words: free radicals, ischemia reperfusion, vitamin C, vitamin E

背景：腎臓缺血-再灌流 (I/R) 損傷可歸因於活性氧 (ROS) 與活性氮 (RNS) 的產生。ROS 導致氧化應激，及隨之而來氧化物 (如 ROS) 與抗氧化物的失衡。本研究旨在比較抗氧化物維生素 C 與維生素 E 對腎臟 I/R 的可能保護作用。

方法：研究材料包括 6 組 Wistar 白化大鼠，分別為：組 1—正常對照；組 2—空白對照；組 3—未經抗氧化物處置的實驗對照。其他 3 組為實驗組，均接受 30 天的維生素處置：組 4—維生素 E；組 5—維生素 C；組 6—維生素 E 及 C 合併。到了第 31 天，除正常對照及空白對照組外，其他組別均接受 60 分鐘腎臟缺血，後續以 10 分鐘再灌流處置。其後研究人員將各組大鼠腎臟摘除，並予以勻漿化，再對腎臟勻漿進行脂質過氧化、還原型腎胱甘肽 (GSH)、及超氧化物歧化酶 (SOD) 等生化檢驗。

結果：在進行腎臟缺血後續再灌流程序後，組織脂質過氧化出現明顯增加，同時 GSH 與 SOD 水平則出現下降 (組 3)。然而，在原先接受維生素 C 及/或 E 的組 4、5、6 的大鼠中，則出現脂質

1Department of Physiology, Center for Basic Sciences, Kasturba Medical College, Bejai, 2Department of Pathology, Kasturba Medical College, Mangalore, and 3Department of Community Medicine, Kasturba Medical College, Manipal, India and 4Department of Physiology, Gharian Medical College, Faculty of Medicine, Al Jabal Al Gharbi University, Gharian, Libya.

Correspondence to: N.A. Vinodini, Department of Physiology, Center for Basic Sciences, Kasturba Medical College, Bejai, Mangalore 575004, Karnataka, India.

E-mail: Vinodini_99@hotmail.com
INTRODUCTION

Ischemia-reperfusion (I/R) injury is a major cause of renal failure and renal graft rejection. Renal I/R injury leads to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. ROS cause oxidative stress, which results in the alteration of the level of mitochondrial oxidative phosphorylation, ATP depletion, increases in intracellular calcium, and activation of protein kinases, phosphatases, proteases, lipases and nucleases leading to a loss of cellular function and integrity [2]. Therefore, it is important to reduce the levels of these hazardous metabolites to improve patient recovery. In order to speed up patients’ recovery from I/R injuries, different types of protection have been suggested and used, including hypothermia, ischemic preconditioning and induction of successive I/R by intermittent clamping for different periods, as well as various drugs used before, during and after the onset of ischemia [3]. Experimental trials have shown the efficacy of these drugs in preventing or attenuating I/R injuries by altering the levels of various agents such as superoxide dismutase (SOD), catalase, mannitol, hypertonic solutions, allopurinol, N-acetylcysteine, iron-binding compounds, angiotensin-converting enzyme inhibitors, calcium channel antagonists, α-tocopherol and ascorbic acid [4–7]. Among them, α-tocopherol is the major component of vitamin E, a group of substances with similar structures. Alpha-tocopherol is considered the most effective lipid soluble antioxidant found in the human biological system. As it interacts with free radicals, the chain reaction of these radicals starts, preventing lipid peroxidation, a process that generates products that are harmful to cells [8].

Ascorbic acid has been used to protect against corneal damage from free radicals in rabbits [9]. In addition, it has also been used to improve the renal hemodynamics and decrease oxidative stress, inflammation and fibrosis in the ischemic kidney of pigs [10]. While previous studies have shown the role of vitamin E in reducing injury, the role of vitamin C has been less extensively studied. Therefore, the present study was designed to investigate the efficacy of ascorbic acid as a free radical oxygen scavenger to attenuate I/R injury and aid in the recovery of renal function.

METHODS

Inbred Wistar rats of either sex, and weighing 200 g, were used in the study. Animals were housed 4–5 rats per cage and fed ad libitum. All experimental protocols were approved by the ethics committee of the Manipal Academy of Higher Education (MAHE) in Manipal, Karnataka, India. The animals were divided into the following groups (7 rats in each group):

• Group 1 (normal control): rats in this group did not undergo ischemia or reperfusion and served as the control group.
• Group 2 (sham control): rats in this group were subjected to the surgical procedure for producing ischemia, but the actual ischemia and reperfusion procedure was not performed.
• Group 3 (untreated experimental control): 60 minutes of renal ischemia were induced in the rats in this group, followed by reperfusion for 10 minutes.
• Group 4 (vitamin E): rats in this group were pretreated with vitamin E 100 mg/kg body weight for 30 days, followed by 60 minutes of ischemia and then 10 minutes of reperfusion.
• Group 5 (vitamin C): rats in this group were pretreated with vitamin C 20 mg/kg body weight for 30 days, followed by 60 minutes of ischemia and then 10 minutes of reperfusion.
• Group 6 (vitamins E+C): rats in this group were pretreated with both vitamins E (100 mg/kg body weight) and C (20 mg/kg body weight) for 30 days, followed by 60 minutes of ischemia and then 10 minutes of reperfusion.

At the end of the experiment, all rats except those in Groups 1 and 2 were anesthetized intraperitoneally with pentobarbitone sodium (40 mg/kg body weight) under strict aseptic conditions. The animals were subjected to left renal vessel occlusion for 60 minutes. After 60 minutes, the vessel occluder was removed to allow reperfusion for 10 minutes. Renal ischemia was confirmed by inspection of the renal vessel, and reperfusion was confirmed by presence of reactive hyperemia. Rats that failed to develop reactive hyperemia were excluded from the study.

The abdominal viscera were covered with gauze soaked in normal saline (0.9% sodium chloride) to keep
the tissues moist. Following completion of ischemia and reperfusion, the kidney was removed and kept in cold phosphate-buffered saline (PBS, 0.9%). The reperfused kidney was blotted dry and minced. The minced tissues were transferred to a glass homogenizer containing 10 mL of cold PBS (pH 7.4) and centrifuged at 3000 rpm for 30 minutes to obtain the supernatant. The obtained supernatant was used for measuring biochemical parameters such as malondialdehyde (MDA), glutathione (GSH) and SOD.

**Estimation of MDA**
MDA was estimated by the method described by Kartha and Krishnamurthy [11]. The development of pink color was measured at 535 nm using a Spectronic D-20 spectrophotometer (Spectronic Devices Ltd., Wootton, Bedfordshire, UK). Thiobarbituric acid-reactive material was expressed in terms of nmol MDA/g wet tissue.

**Estimation of GSH**
GSH was estimated using the standard protocol of Beutler et al [12]. The optical density of the blank test tube with all the reagents except the samples and the test tube with all the reagents and the sample was measured at 412 nm using a Spectronic D-20 spectrophotometer (Spectronic Devices Ltd.). The GSH content of tissue was expressed as μg GSH/g tissue protein.

**Estimation of SOD**
SOD was estimated by the technique described by Beauchamp and Fridovich [13]. The reduction of nitroblue tetrazolium by O$_{2}^{-}$ was measured at 560 nm using a Spectronic D-20 spectrophotometer (Spectronic Devices Ltd.). The activity was expressed as unit/mg protein.

**Protein estimation**
The protein content of the tissue samples was determined by the method of Lowry et al [14]. The blue color developed was measured at 540 nm using a Spectronic D-20 spectrophotometer (Spectronic Devices Ltd.).

**Statistical analysis**
Data were analyzed using the non-parametric Kruskal–Wallis test followed by multiple comparison tests, with $p < 0.05$ considered to be significant. Data are expressed as mean ± standard deviation.

**RESULTS**
When the rats were pretreated with the antioxidants vitamin E and vitamin C alone (Groups 4 and 5), and with both vitamins E and C (Group 6), a significant decrease in tissue lipid peroxidation (Figure 1), an increase in GSH (Figure 2) and an increase in SOD (Figure 3) were observed when compared to the untreated experimental control (Group 3; $p < 0.0001$). However, there were no statistical differences among the treated groups (Groups 4, 5, 6), or among the control groups (Groups 1 and 2).

**DISCUSSION**
Renal ischemia initiates a complex and interrelated sequence of events, resulting in the injury and death of renal cells [15]. Reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage to the same tissue that is referred to as reperfusion injury [16]. Together, I/R of the kidney contribute to the
renal dysfunction and injury associated with ischemic acute renal failure [17,18]. Although the exact mechanisms involved in the pathogenesis of acute renal failure have not been fully elucidated, it is generally believed that ROS and RNS are the key mediators of I/R-induced kidney damage [19]. One of the approaches to limiting apoptotic or necrotic cell death in response to I/R injury may be antioxidant therapy. Antioxidants such as vitamin E have been shown to have protective properties against ischemia-induced tissue damage [20,21]. The results of the present study showed a decrease in tissue MDA level and an increase in scavenging enzymes such as SOD and GSH upon treatment with either vitamin C and E or both following I/R. The results of this study suggest that the levels of various antioxidant enzymes (SOD, GSH), which protect against oxygen free radicals, were higher in the rats treated with vitamin C. Ascorbic acid reduces the level of reactive oxidant species both intracellularly and extracellularly, and maintains transition metals in their reduced form [1,22]. Thus, the present study indicates that ascorbic acid as an exogenous antioxidant may attenuate I/R injury by increasing the activities of SOD, GSH peroxidase, and catalase. There is evidence to suggest that vitamin C may affect intracellular levels of GSH, which can improve flow-mediated, endothelium-dependent dilation [23]. When endogenous lipid peroxidation was measured, it was decreased by vitamin C administration in most

Figure 2. Effect of pretreatment with vitamin C on tissue glutathione (GSH) following renal ischemia-reperfusion. Data are expressed as mean±standard deviation. *Group 3 vs. Groups 1, 2, 4, 5, 6, p ≤ 0.0001. Group 2 vs. Group 1 is not significant (NS).

Figure 3. Effect of pretreatment with vitamin C on tissue superoxide dismutase (SOD) following renal ischemia-reperfusion. Data are expressed as mean±standard deviation. *Group 3 vs. Groups 1, 2, 4, 5, 6, p ≤ 0.0001. Group 2 vs. Group 1 is not significant (NS).
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