Critical time point for apoptotic cell death in an experimental ischemia/reperfusion model and the effect of N-acetylcystein

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Abstract

Objective: Kidney transplantation is an important treatment option in end stage renal failure. Tissue death may be an important problem when a kidney is removed from a cadaver and transported to a donor a long distance away. The purpose of this study is to determine the critical time point for apoptotic cell death in a renal ischemia/reperfusion model and determine the effects of N-acetylcystein on apoptosis induced by ischemia injury.

Methods: Apoptotic cell death after induced renal ischemia followed by reperfusion, was estimated in a group of Wistar albino rats by immunofluorescence and ELISA techniques. N-acetylcystein, an antioxidant agent, was given to the rats to study the effect on apoptosis. Tissues were examined immunohistochemically at 0, 1 h, 24 h, 5 days and 10 days for detection of apoptotic cells.

Results: Our results showed that an ischemia for 60 min followed by reperfusion for 60 min triggered apoptosis. Moreover, N-acetylcystein significantly diminished both the ischemia/reperfusion damage and apoptosis.

Conclusion: We anticipate our results would be important for kidney transplantation in estimating the critical time point for apoptosis and administration of N-acetylcystein prior to removal of the organ may be important in delaying the onset of apoptosis.

Keywords: Kidney; Transplantation; Ischemia/reperfusion; Apoptosis; N-acetylcystein.

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Özet

Amaç: Böbrek nakli son dönemde kronik böbrek yetmezliğinde önemli bir tedavi seçeneğidir. Kadavradan böbrek çıkarılıp donöre takılmak üzere uzun mesafelere nakledildiğinde doku ölümü önemli bir sorun olabilir. Bu çalışmanın amacı renal iskemi reperfüzyon modelinde apoptotik hücre ölümü için kritik zaman aralığını saptalamak ve N-asetilsistein’in iskemik hasara karşı olan etkisini göstermektedir.

Yöntem: Renal iskemi-reperfüzyon sonrası apoptotik hücre ölümü Wistar albino çıçanlarında immunofloresans ve ELISA yöntemleriyle gösterilmiştir. Deneklere apoptoz üzerindeki etkinin gösterilmesini amacıyla bir antioksidan ajan olan N-asetilsistein verilmiştir. Dokular apoptotik hücrelerin gösterilmesi için 0, 1. saat, 24. saat, 5. gün ve 10. günlerde immunohistokimyasal olarak incelenmiştir.

Bulgular: Sonuçlarımız 60 dakikalık iskemiyi takiben 60 dakikalık reperfüzyon yapılmışın apoptozu
Introduction

Ischemia (I) defined as depletion of $O_2$ required for the maintenance of tissue aerobic metabolism and reperfusion (R) is defined as reinstitution of oxygenated blood to the previously deprived tissue [1, 2]. During ischemia, pro-inflammatory cytokines are released and activate innate immunity which is a cause of free radical-mediated injury. Reperfusion, on the other hand, results in inflammation, apoptosis and cell death possibly as a result of oxidative stress. Hypoxia initiates a series of biochemical reactions that end up with cellular dysfunction and if prolonged leads to ultimate cell death via diminishing cellular energy level and accumulating toxic substances [3–5]. Prolonged ischemia results in destruction of cellular integrity leading to necrotic cell death, whereas in animal models with shorter periods of ischemia, apoptosis is the primary mode of cell death [6].

Ischemia/reperfusion injury (IRI) is caused by restriction of blood supply to the particular organ followed by restoration of blood flow and re-oxygenation [2]. Although the exact mechanism involved in the pathogenesis of renal I/R injury has not been fully understood, it is generally believed that the reactive oxygen and nitrogen species (ROS and RNS, respectively) are the key mediators of I/R-induced damage to the kidney [7–11]. Reactive oxygen species cause renal cell injury by oxidation of proteins, peroxidation of lipids, damage to DNA and induction of apoptosis. Reactive oxygen molecule scavengers such as superoxide dismutase, catalase and N-acetylcysteine protect ischemic renal cell injury [12, 13]. N-acetylcysteine (NAC), which has a thiol group that is a source of L-cysteine and reduced glutathione, serves as an antioxidant interacting with reactive oxygen species [14, 15].

Kidney transplantation is an important treatment option in end stage renal failure. Oxidative stress is generated when an imbalance exists between oxidants and antioxidants and it is an important parameter for the success of transplantation [16, 17].

The antioxidant actions of NAC during and after ischemia-reperfusion (I/R) injury have been studied in experimental animals and in human beings after renal transplantation [14–18]. However, the effect of NAC on apoptosis induced in I/R injury has not been fully understood. The purpose of this study is to determine the significant time intervals which are critical for renal I/R injury and determine the effects of NAC on apoptosis induced in I/R injury.

Materials and methods

Animals and tissue preparation

The animal experiments were approved by the Ethics Committee for animal research of the University of Çukurova (27.01.2009/4) and carried out in accordance with guidelines described by the committee. A total of 35 age-matched male Wistar albino rats (220–260 g) were employed in the study. Ischemia was developed in the left kidneys using a traumatic vascular clamp to block blood flow and reperfused via removal of clamps following ischemia for 60 min. Around 15 mg of tissue samples were excised from the reperfused left (I/R, n = 4) and untreated right (C, n = 4) kidneys at 0, 1 h, 24 h, 5 days and 10 days time intervals for apoptotic analyses. The other group of rats (n = 4) were injected with 72 mg/kg NAC 2 h before the ischemia and analysed under the same conditions to compare with untreated tissue samples. The rats were then sacrificed and their kidneys were isolated. Half of the kidney tissues were fixed in 10% formaldehyde for the histopathologic and immunohistochemical analyses. The other half was kept in −80°C until the apoptotic measurements.

Histopathological analyses

Half of the kidney tissues, fixed in 10% formaldehyde, were stained by hematoxylin and eosin (H&E) for the histopathologic analyses. Five micron thick samples were evaluated under the light microscope (Nikon E600, Tokyo, Japan) by a single pathologist blinded to the groups and treatment conditions. Tubular injury was identified as tubular epithelial necrosis due to swelling and vacuolization of the tubular epithelial cells, sloughing of the tubular cells into the tubular lumens and intratubular debris.
Immunohistochemistry

The apoptotic cells in rat kidneys were detected by using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (ApopTag® S7101, Chemicon International, Inc.). The kit detects apoptotic cells by labeling and detecting DNA strand breaks by the indirect TUNEL method, which labels the free 3′-OH termini with modified nucleotides. ApopTag positive nuclei of tubular cells were counted on 10 different areas, at ×400 magnification. The mean number of ApopTag-positive cells was used for the statistical analysis.

Flow cytometry

Frozen kidney samples were cut into 1 mm³ pieces and washed in ice cold phosphate buffer saline (PBS). Then the samples were incubated with buffer containing 1mg/mL trypsin in 37°C for 50 min. At the end of the incubation period the cells were centrifuged at 200 × g for 5 min and the supernatant was discarded. The cells were resuspended in 1–2 mL PBS, recentrifuged and washed three times with PBS. In order to detect the apoptotic cell percent 100 μL cell suspensions were incubated with Annexin V-FITC and incubated on ice in the dark for 10 min. After incubation, 400 μL of binding buffer was added. The samples were analyzed by a FACS flow cytometer (Epics-XL Beckman-Coulter).

Measurement of apoptotic cell death

Tissue samples were homogenized and washed using 200 μL of Phosphate-buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) to remove the blood and centrifugated (5 min at 200 × g) to obtain the suspended cells from upper phase. Centrifugation was repeated at 11,337 × g through 5 min and cell-free upper phase were removed while cell pellets were fixed with 100 μL of 80% methanol in PBS. Single stranded-DNA apoptosis ELISA kit was used in measurement of apoptotic cell percentage according to manufacturer’s specifications (Chemicon-Millipore, Billerica, MA, USA). The principle of the method was based on the selective denaturation of apoptotic DNA using formamide followed by the detection of DNA ladders using monoclonal antibody.

Statistical evaluation

One way analysis of variance (ANOVA) was used in comparisons between the measured apoptosis absorbance values among different groups at 95% confidence interval.

Results and discussion

Renal ischemia-reperfusion (I/R) injury is the primary cause of acute kidney damage. This condition usually occurs during hemorrhagic shock, sepsis, renal transplantation, partial nephrectomy, and urological surgery procedures requiring clamping of the renal artery. Renal I/R injury may disturb tubular structure and kidney functions and due to the malfunctions in the kidney, the patients’ prognosis becomes worse after an acute kidney damage. Clinically, it can lead to prolonged hospitalization or even organ loss [19–22].

Although the molecular mechanisms of renal I/R injury are not completely understood; oxidative stress is a well-recognized pathogenic factor [16]. Reactive oxygen species (ROS) are produced primarily by mitochondria and cells have several antioxidant defense mechanisms, such as superoxide dismutase and glutathione. Excess reactive oxygen species (ROS) in cells induce oxidative damage of lipid membranes and biological macromolecules and generate tissue damage by apoptosis and necrosis [22–24].

It is generally believed that ROS and reactive nitrogen species (RNS) play the pivotal role in the mediation of I/R. Several agents such as melatonin [7], vitamin E (α-tocopherol) [8], erdosteine [8], EPC-K1 [20], 2-aminoethyl diphenylborinate [21], dexamphenol [25], syringic acid [26], humic acid [27], vitamin C (ascorbic acid) [28], atorvastatin [29] ethyl pyruvate [30], sodium nitroprusside [31] and phosphormidon [31] have been shown to have protective properties against ischemia-induced tissue damage. The protective effect of NAC, an antioxidant interacting with reactive oxygen species has been investigated by several groups either alone [14, 18, 19, 22, 32–35] or combined with other agents [25, 29–31].

Although the protective role of NAC for renal I/R injury has been experimentally demonstrated by other investigators, little is known about the critical time point and the protective effect of NAC for apoptotic cell death in renal I/R injury, which is a very important parameter for kidney transplantation.

Therefore we aimed to identify the effective parameters in I/R models in an attempt to develop effective treatment strategies for acute kidney failure and estimate critical time parameters for organ transplantation. We focused on the comparison of proliferative, necrotic and apoptotic cell death in an experimental kidney ischemia model followed by reperfusion via O₂ supply following ischemia in experimental animals. Because of the known effects of anti-oxidative agents in reversing a number of pathological cases, including kidney failure, we also analyzed the effects of the antioxidant agent NAC on apoptotic...
cell death and evaluated the protective role of NAC on kidney injury in our experimental model.

We chose the parenteral route and no side effects were detected in our study. Rats were injected with 72 mg/kg NAC 2 h before the ischemia and analysed under the same conditions for comparing to untreated tissue samples.

We first determined the apoptotic cell percentage among the samples by means of a selective denaturation procedure of apoptotic DNA. We monitored DNA ladders using a monoclonal antibody and found that the apoptotic cell percentage did not significantly differ between I/R and control at 0 h between the right and left kidneys before I/R injury (Figure 1A). However the samples following 60 min after I/R revealed a significantly higher percentage of apoptotic cell death compared to the control group (n = 4, p < 0.05, Student t-test) while the samples of the experimental group analyzed following 24 h, 5 days and 10 days did not reveal a significant difference in apoptotic cell death (data not shown). Since 60 min is a very short time point we did not need to monitor renal functions.

On the other hand, when the left (I/R) and control right kidney samples were analysed using Tukey HSD multiple comparison tests, the samples of 60 min of I/R application gave rise to significantly different results when compared to the rest of the samples comprising 24 h, 1 day and 5 days (n = 4, p < 0.05, one way ANOVA, Tukey HSD multiple comparison post hoc test) (Figure 2).

However, the effect of NAC on apoptosis induced in I/R injury has not been fully understood. Therefore we included NAC 2 h before the ischemia and then analysed the tissue samples. Sixty minutes of I/R significantly increased the apoptotic cell percentage (p < 0.05) compared to the control group and NAC injection before I/R significantly reversed this effect (n = 4, p < 0.05, One way ANOVA, Tukey HSD multiple comparison post hoc test) (Figure 3).

We determined apoptotic cell death using Annexin V as an apoptotic cell marker. The apoptotic cell death detected with Annexin V by flow cytometry revealed that apoptotic cell percentage was higher in the 60 min I/R group compared to the control group (p < 0.01). As seen in Figures 4 and 5, NAC injection significantly reversed this effect (p < 0.01) in the same time period (n = 4, p < 0.01, One way ANOVA, Tukey HSD multiple comparison post hoc test) (Figures 4 and 5).

We compared the tissue samples upon histopathological examinations. The samples did not reveal major difference from each other in terms of histomorphology under light microscope (data not shown).

Finally the apoptotic cells were detected by using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit. In the 60 min of I/R group the apoptotic stained cells were

![Figure 1: Apoptotic cell percentage among the samples, covered in this study. (A) The apoptotic cell percentage between I/R and control at 0 h detected with ELISA (n = 4). (B) The apoptotic cell percentage between I/R and control at 60 min (n = 4), *p < 0.05.](image1)

![Figure 2: Apoptosis in the left (I/R) and control (N) right kidney samples in whole groups detected with ELISA. *p < 0.05.](image2)
visible whereas there was a remarkable decrease in apoptotic cells in NAC group (Figure 6).

Previously, several studies have revealed the protective effects of antioxidants on apoptotic cell percentage. For example in a recent study by Sancak et al. syringic acid decreased apoptosis significantly when administered before I/R [26]. Likewise, Akbas et al. found that humic acid, which is a polyphenolic antioxidant, diminished the apoptotic cells when administered before I/R in immunohistochemically stained samples with ApopTag which was also used in our study as well [27].

Moreira et al. studied the effect of NAC on renal ischemia/reperfusion injury induced by hemorrhagic shock (HS) and subsequent fluid resuscitation (HS/R) which is a common cause of acute renal failure. Animals treated with NAC presented attenuation of histologic lesions, reduced oxidative stress and apoptosis markers when compared with animals from the HS/R group [35].

Zu et al. found that NAC reduced the expression of a mitochondrial protein regulating the apoptotic marker Bax in a model of cardiac IR and suggested that apoptotic cell death is related to increased ROS production during IR, and NAC has an essential role in mediating IR induced injury by inhibiting ROS [36].

We showed that 72 mg/kg NAC injection 2 h before the renal ischemia can have protective effect on ischemia-induced damage and reduce apoptotic cell death. Azarkissh et al. evaluated the protective role of low (150 mg/kg) and high (500 mg/kg) doses of NAC on kidney injury and distant lung injury following bilateral renal I/R injury. The low-dose of NAC was more effective than the high dose in protecting against the renal I/R injury and

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**Figure 3:** The effect of N-acetylcysteine (NAC) on apoptosis detected with ELISA.
The left (I/R) and control (N) right kidney samples in both control and NAC groups at 60 min, n=4, *p < 0.05, one way ANOVA, Tukey HSD multiple comparison post hoc test.

**Figure 4:** Results of fluorescent microscopic analyses of the cells, painted with Annexin V-FITC and propidium iodide.
The impact of N-acetylcysteine on distribution of living (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells was analysed in control groups, control + NAC groups, and groups to which I/R was applied. At least 10,000 cells were analyzed per sample in quadrant.
administration of an antioxidant after kidney I/R injury may protect not only the kidney but also the distant organ from the ischemia-induced damage [22].

Fuller et al. evaluated the influence of donor pre-treatment with N-acetylcysteine on ischemia/reperfusion injury in rat kidney grafts and suggested that in a rat kidney transplantation model with moderate I/R injury donor pretreatment with NAC preserves renal metabolism [37].

Few studies have addressed the effects of NAC in transplant recipients. Danilovic et al. suggested that attenuation of oxidative stress by NAC administration to recipients of deceased donor renal transplant promoted faster and sustained recovery of allograft function. Recipients who received NAC showed faster, sustained allograft functional recovery [19].

NAC also has protective effects on renal I/R injury in an electrical burn model [30] and hemorrhagic shock [35]. Turkmen et al. concluded that NAC or ethyl pyruvate therapy can be effective for the purpose of reducing tissue injury occurring after exposure to electricity [30].

In this study, N-acetylcysteine (NAC), a low-cost drug with low rate of adverse effects, is used as an antioxidant agent. Taken together, our results suggest that an ischemia with a 60 min duration followed by a 60 min of reperfusion triggered apoptosis. Moreover, we evaluated the protective role of NAC on kidney injury and found that NAC provides remarkable protection on kidney injury. NAC significantly diminished both the ischemia/reperfusion damage and apoptosis. Our findings have potential importance in clinics because the 60 min duration was designated as a critical period for kidney surgical operations.

**Conclusion**

In conclusion, our study suggested that NAC pretreatment 2 h before operation reduces apoptotic cell death caused by ischemia/reperfusion injury during kidney surgery. The critical timepoint for apoptotic cell death is 60 min in kidneys submitted to IR injury. In kidney transplantations donor pretreatment with NAC might be beneficial in limiting tissue damage and reducing apoptotic cell death.

**Figure 5:** Apoptotic cell death detected with Annexin V by flow cytometry, n = 4, **p < 0.01 I/R control vs. I/R 60 min, ***p < 0.01, (I/R) 60 min vs. (NAC + I/R) 60 min, one way ANOVA, Tukey HSD multiple comparison post hoc test.

**Figure 6:** The apoptotic cells in rat kidneys detected by using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (×400 magnification). (A) I/R 60 min. (B) N-acetylcysteine 60 min. following I/R 60 min.
Conflict of interest statement: The authors declare no conflict of interest.

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