Protective effects of amifostine on ischemia-reperfusion injury of rat kidneys

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ABSTRACT

Objectives: Amifostine is a drug which can eliminate free oxygen radicals that appear in the body after radiation or chemotherapeutic agent exposure. It is used to decrease the renal toxicity of cisplatin. The aim of this study was to determine the role of amifostine in warm ischemia kidney model for prevention of ischemia/reperfusion injury and also to find out the mechanism for prevention from ischemia/reperfusion injury if such an effect does exist.

Materials and Methods: Adult female rats \( n = 40 \) that used in our study were divided into three groups. Group 1: Control \( n = 8 \), group 2: Ischemia-control \( n = 16 \), group 3: Amifostine treated \( n = 16 \). The effect of amifostine on ischemia/reperfusion injury investigated in rat kidneys.

Results: At the 7th day, blood urea nitrogen level was statistically significantly higher in ischemia-control group than all groups \( P = 0.001 \) and mean serum creatinine levels were found to be the highest in ischemia-control group \( P = 0.091 \). Mean malondialdehyde levels in left kidneys removed on the 7th day were not significantly different \( P = 0.105 \) at all three groups. Between ischemia-control group and amifostine group, there was a significant difference in reduced glutathione (GSH) levels \( P = 0.001 \). In amifostine group, grade 4 necrosis was not detected neither on 7th day nor day 0.

Conclusion: Amifostine could decrease the degree and severity of necrosis after reperfusion. Amifostine could not prevent membrane lipid peroxidation caused by superoxide anion radicals in kidney but they could protect tissues from the harmful effects of ischemia/reperfusion injury by increasing the level of reduced GSH which is a well-known oxygen radical eliminator.

KEY WORDS: Amifostine, ischemia-reperfusion injury, kidney

Introduction

With reperfusion of tissues following reduced or ceased blood flow, molecular oxygen delivery to the tissue can cause irreversible cell damage.\(^{[1]}\) This condition can be encountered during thromboembolic processes in myocardial or brain tissues, hypovolemic shock, sepsis, cardiac arrest, resuscitation, and organ transplantation.\(^{[2-3]}\) Damage caused by reperfusion after ischemia is more harmful than that of ischemia causes itself.\(^{[4]}\)

It has been shown by many clinical and experimental researches that the basic mechanism of tissue damage due to ischemia-reperfusion (I/R) is the increase at the level of free radicals after reperfusion.\(^{[7,8]}\) It has been shown that the free radicals destroy cells in every kind of tissues and alters the gene function. The activation of complement system, production of chemotactic peptides, migration and activation of leukocytes, membrane and lipid changes due to free oxygen radicals and intracellular depletion of free radical eliminators cause reperfusion injury.

Amifostine (ethiole) eliminates free oxygen radicals produced after radiation or some chemotherapeutic agents.\(^{[9]}\) In clinical practice, it is used in order to decrease the renal toxicity of cisplatin and to lessen xerostomia caused by radiotherapy for head and neck cancers.\(^{[10,11]}\) The active metabolite of amifostine thiol, WR 1065, is a fast eliminator of free oxygen radicals, detoxifies the active forms of alkylating agents by directly blocking them intracellularly. It
provides chemical repair of deoxyribonucleic acid (DNA) by transferring a hydrogen atom to DNA.\textsuperscript{12} Because amifostine has eliminative effect on free oxygen radicals, it can have a protective effect on I/R injury. A previous study has shown that amifostine prevented I/R injury through eliminating free oxygen radicals in rabbit spinal cord ischemia injury model.\textsuperscript{13} Another study has investigated the effect of amifostine in rat renal I/R injury model but effect of amifostine on glutathione (GSH) peroxidase or other antioxidant enzymes could not be detected.\textsuperscript{14}

The aim of this study was to determine the role of amifostine in warm ischemia kidney model for prevention of I/R injury and also to find out the mechanism of prevention from I/R injury whether though antioxidant or not if such an effect does exist.

Materials and Methods

Study Design

This study was approved by the Animal Care Committee of Akdeniz University, Turkey. The study was carried out with 40 young adult rats weighing between 250 and 300 g. The animal room was maintained at a temperature of 23°C ± 3°C and a relative humidity of 65% ± 15%. Water and chow were freely available throughout study periods.

All the rats were nephrectomized on the right side, and renal functions of all the rats turned out to be dependent upon their left kidneys. The rats were divided into three groups.

- **Group 1 (control):** Sham operation done group (\(n = 8\)). In this group of rats, femoral catheterization and right nephrectomy were done only. No treatment was given.
- **Group 2 (ischemia-control):** Nontreated ischemic kidney group (\(n = 16\)). In this group, rats were operated with a method which as described below.
- **Group 3 (amifostine):** Amifostine-treated ischemic kidney group (\(n = 16\)). The same operation as with the second group was performed. Amifostine with a dose of 30 mg/kg was injected intravenously in 2 mL solution.

Surgical Procedure

Young adult female rats were given intramuscular ketamine hydrochloride in a dose of 35 mg/kg and combined with mild ether anesthesia. They were placed on the operation table in the supine position and stabilized on the extremities. Before the experiment, 0.3–0.4 mL of blood specimens were driven from all of the rats to analyze blood urea nitrogen (BUN) and creatinine levels. After laparotomy with midline incision, left renal vascular pedicle was isolated. Heparin (0.5 mL 80 IU) was administered through femoral cannula to all rats. After heparinization, 0.9% saline, 2 mL was administered to rats in group 1 and 2. Group 3 were given 2 mL amifostine solution containing 30 mg/kg amifostine in 2.0 mL of 0.9% saline. Afterward, renal pedicle was occluded with an atraumatic clamp. Occlusion was not done in sham group. Abdomen was closed temporarily with 3/0 running silk suture in order to minimize fluid loss. After 45 min, abdomen was reopened, and clamp was removed. Kidney was observed for 4 min, and if the circulation was not provided in 2 min, the rat was excluded from the study assuming that there is vascular thrombosis. Right nephrectomy was done immediately and in order to supply fluid loss 0.9% saline was given with a dose of 2 mL/100 g intraperitoneally. The abdomen was closed with 3/0 running silk suture in two layers. Six rats from each group except sham group were sacrificed after 5 min following reperfusion and left nephrectomy was done, and kidneys saved for both immunohistopathological investigation and GSH level determinations. Other rats were observed for the following 7 days. On the 3rd and 7th days, blood was driven from the tail vein for BUN and creatinine analysis. On the 7th day, under anesthesia, left nephrectomy was performed. The half of the removed kidney material, which were removed after 5 min and after 7 days of reperfusion was put in formaldehyde solution, and histopathological investigation was carried out blindly. The other remaining left half of the kidney material was frozen in nitrous oxide and kept at −70°C for later determination of malondialdehyde (MDA) and GSH levels.

Histopathological Investigation

Histopathological investigation was done with regard to the necrosis grading method on rat kidneys described by Jablonski et al.\textsuperscript{15} [Table 1]. Immunological study was carried out with immunoglobulin G, immunoglobulin M (IgM), immunoglobulin A, CDB and NK stains. After staining of cells, a scoring system composed of the percentage of stained cell number and intensity of staining was used for final analysis as follows:

- **Percentage of stained cells**
  - Point: 0 (0% stained cell)
  - Point: 1 (less than 25% stained cell)
  - Point: 2 (between 25% and 75% stained cell)
  - Point: 3 (more than 75% stained cell)
- **Intensity of being stained**
  - Point: 0 (no staining)
  - Point: 1 (mild)
  - Point: 2 (moderate)
  - Point: 3 (intense).

Statistical Analysis

Statistical evaluations were performed using SPSS 18.0 (SPSS, Chicago, USA). The group level differences of BUN, creatinine, MDA, GSH levels were evaluated with “Kruskal–Wallis test” and for comparing the intergroup mean levels “Mann–Whitney U-test” was used. The results of histopathological examinations were classified as positivity and negativity and for comparison “Chi-square test” was used. If the expected level was lower than 5, “Chi-square test” was used. For overall statistical evaluation, “\(P\)” levels <0.05 were regarded as significant.

### Table 1:

| Grade    | Description                                      |
|----------|--------------------------------------------------|
| Grade 0  | Normal                                           |
| Grade 1  | Mitosis and necrosis in some of the cells         |
| Grade 2  | Necrosis present in cells neighboring to the vital proximal convoluted tubules |
| Grade 3  | Necrosis at the distal 1/3 of proximal convoluted tubules |
| Grade 4  | Necrosis at all of each three segments of the proximal convoluted tubules |
Results

The Mean Blood Urea Nitrogen Levels in Rats that Have Been Kept Alive for 7 Days

In all groups, 3rd and 7th day BUN mean levels were higher than that of the 1st day levels, and this was statistically significant. At the 7th day, BUN level of ischemia-control group was higher than that of all groups which is statistically significant (P = 0.001) [Table 2].

Mean Creatinine Levels in Rats that are Vital for 7 Days

On 3rd day, serum creatinine levels in amifostine group were higher than those that were measured at 1st and 7th days (P = 0.005).

In all three groups, serum mean creatinine levels at the 3rd and 7th day were found to be indifferent (respectively, P = 0.007, P = 0.001). Seventh-day serum mean creatinine levels were found to be the highest in ischemia-control group [Figure 1].

Mean Malondialdehyde Levels in the Kidneys Removed on 7th Day and Day 0

Mean MDA levels in kidneys of alive rats in all three groups on the 7th day were not statistically significantly different (P = 0.105) and also on the day 0, MDA levels in kidneys of sacrificed rats were not statistically significantly different (P = 0.211).

Mean Glutathione Levels in Kidneys that were Removed on the 7th Day and Day 0

Mean GSH levels in kidneys of alive rats in all three groups on the 7th day were evaluated, levels were statistically significantly different (P = 0.001). In the control group, mean GSH level was 85.58 nmol/g, meanwhile level was decreased significantly in ischemia-control group (66.05 nmol/g) (P = 0.010). Furthermore, there was a statistically significant difference between ischemia-control group and amifostine group (P = 0.000). On day 0, the lowest level was in ischemia-control group (60.86 mmol/g), the highest level was in amifostine group (104.46 mmol/g).

Immunohistopathological Evaluation

In the control group, necrosis was not encountered in any of the 8 kidneys removed on the 7th day. In ischemia-control group, grade 2 necrosis was encountered in 3 of the 6 kidneys removed on the day 0. At the other remaining 3 kidneys, grade 1, 3, and 4 necrosis was detected. On the 7th postoperative day, grade 1 and 2 necrosis in 5 kidneys, grade 3 and 4 necrosis in the other 5 kidneys was detected in ischemia-control group [Figure 2]. Grade 4 necrosis was not detected in kidneys of amifostine groups on 7th day and day 0. After staining of all kidneys that were removed on the 7th postoperative day, evaluation with respect to necrosis grade yield that there was a statistical significant difference (P < 0.05) [Table 3].

There was a statistically significant difference between groups according to CD8 staining of kidneys removed at day 0 (P = 0.0125). Among ischemia-control group, four kidneys contain CD8 stained lymphocytes and in two kidneys staining was not occurred [Figure 3]. Among treatment given group, CD8 stained lymphocytes were not detected in any kidney.

Kidneys, which were removed on 7th day, stained with IgM stain, only one kidney in the control group had positive stain.

Table 2:
The mean BUN levels in 7 days alive rats (mg/dL)

| Groups          | BUN 1 | BUN 3 | BUN 7 | P     |
|-----------------|-------|-------|-------|-------|
| Control         | 23.8±6.94 | 31.8±4.59 | 35.8±5.26 | 0.002 |
| Ischemia-control| 22.8±3.16 | 37.4±4.77 | 43.0±3.33 | 1     |
| Amifostine      | 24.2±3.36 | 35.0±3.43 | 35.0±3.80 | 0.002 |
| P               | 0.060 | 0.110 |       | 1     |

BUN=Blood urea nitrogen

Figure 1: The mean creatinine levels in 7-day alive rats

Table 3:
Necrose grading of H and E stained kidneys removed from rats at postoperative 0 and 7 days

| Histopathologic grade | Postoperative 0 day | Postoperative 7th days |
|-----------------------|---------------------|------------------------|
|                       | 0 1 2 3 4 n         | 0 1 2 3 4 n            |
| Control               | 0 0 0 0 0 8         | 0 0 0 0 8              |
| Ischemia-control      | 0 1 3 1 1 6 0      | 3 2 3 2 10             |
| Amifostine            | 2 2 2 0 0 6 3      | 5 1 1 0 10             |

Discussion

Reperfusion injury in the kidney increases morbidity and mortality especially after transplantation, aorta, and open heart surgery. Although it was shown that some medicines are effective in preventing ischemia-reperfusion injury, we have very few data indicating the effects of amifostine in this regard.

In this study, at the 1st, 3rd and 7th day in all groups, there was an increase in BUN levels (P < 0.05), but in treatment given group, amount of increase was not high as ischemia-control group. In the treatment given group, there was an increase in creatinine levels on the 3rd day but on the 7th day decline was observed [Table 2]. These results indicate that amifostine plays a preventive role on I/R injury.

Spencer and Goa investigated amifostine and consequently showed that WR 1065, the active metabolite of WR 2721,
played a protective role in rat heart injury model caused by chemotherapeutic drugs in vitro and also it provided increase in intracellular GSH levels (33–%74). In an experimental study done by Pissarek et al. shown that WR 2721 served as a free radical eliminating agent, and it had protective potential in reperfused heart model. In this study, it is shown that WR 2721 diminished the exposition rate of free radicals that are produced endogenously in reperfused heart rat model.[18] In our study, likewise Pissarek et al.’s study, amifostine group GSH levels were increased. This finding indicates that amifostine causes decrease in free radical levels in the kidney. Although there are a lot of studies about the protective effect of amifostine on cisplatin toxicity, there are only 2 studies investigating the effect of amifostine on I/R injury. One study showed that amifostine attenuates spinal cord oxidative injury in rabbit models.[13] In this study, 43% reduction rate for free oxygen radicals, 38% reduction rate for lipid peroxidation markers after amifostine treatment in rabbit spinal cord injury model were detected. Other study resembling our study showed that amifostine decreases tubular injury after reperfusion in rat kidney models. In this study, decrease in levels of MDA, increase in expression of cyclooxygenase-2 were detected in two groups that are treated with amifostine when compared with control group, but there was not any significant difference at GSH peroxidase and other antioxidant enzyme levels.[14]

In our study, there was no any statistically significant difference between all groups on day 0 and 7th day according to tissue MDA levels which increases as a result of membrane lipid peroxidation. In our experience, I/R injury did not cause any rise in membrane lipid products. This result is in contrary with Chok et al.’s and Chronidou et al.’s findings. GSH, one of the tissue antioxidant defense mechanisms, levels were low in ischemia-control group, but on the contrary levels were high in amifostine administered group on day 0 and 7th day ($P < 0.05$). Thus, amifostine can prevent I/R injury in the kidney. Since we could not find significant variations in MDA levels, its preventive effect may not be via inhibiting membrane lipid peroxidation but may be with different mechanisms. The increase in GSH, serving as an oxygen radical eliminator that exists in the organism, indicates that amifostine activates this mechanism separately. In ischemia-control group, necrosis was shown immuno-histopathologically as well. It has been found that amifostine therapy could prevent necrosis. Among the parameters studied, only in CD8 cells obvious response to reperfusion was detected and has been shown that this response could be prevented by amifostine.

In this study, it was concluded that amifostine can protect tissues from the harmful effects of I/R injury. Amifostine exposes this effect by increasing the level of reduced GSH, which is a well-known oxygen radical eliminator. Positive results achieved from few number of studies about the effect of amifostine on I/R injury give us hope about usage of amifostine in clinical cases for preventing I/R injury in the future.

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