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An Animal model to determine the role of amifostine in prevention of ischemia/reperfusion injury: A quasi-experimental study

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Abstract
Aim: to determine the role of amifostine in prevention of ischemia/reperfusion injury.
Materials and Methods: 30 adult rats were divided into three groups. Group I: Control (n=6), group II: Ischemia-control (n = 12), group III: Amifostine treated (n = 12). SPSS version 20 (SPSS Inc., Chicago, Illinois, USA).
Results: At the 7th day, BUN level was found statistically significantly higher in group II than rest two groups ($p<0.05$) and mean serum creatinine levels were found to be the highest in group II ($p<0.05$). Between group II and III, there was a significant difference in reduced glutathione (GSH) levels ($p<0.05$).
Conclusion: Amifostine could decrease the degree and severity of necrosis after reperfusion.

Keywords: amifostine, BUN, creatinine, GSH, Ischemia-reperfusion injury

Introduction

condition can be encountered during thromboembolic processes in myocardial or brain tissues, hypovolemic shock, sepsis, cardiac arrest, resuscitation, and organ transplantation [1, 2]. Damage caused by reperfusion after ischemia is more harmful than that of ischemia causes itself [3].

The complete mechanisms underlying IR injury are complex and incompletely understood, but oxidative stress, necrosis, cell apoptosis, ATP depletion, and calcium dyshomeostasis all contribute to the mechanisms of renal IR injury [4, 5]. The generation of reactive oxygen species (ROS) in the reperfusion phase leads to DNA mutation and initiation of apoptotic and necrotic death cascades, ultimately leading to cell death [6]. Suppressing IR injury would improve outcomes in any clinical setting in which IR injury is encountered, including transplantation, vascular surgery, urologic surgery, and neurosurgery.

Amifostine (ethiole) eliminates free oxygen radicals produced after radiation or some chemotherapeutic agents [7]. 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 [8, 9]. 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 [10]. Because amifostine has eliminative effect on free oxygen radicals, it can have a protective effect on I/R injury.

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

A Prospective quasi experimental study was conducted for the period of Dec 2012 to August 2013 among 30 young adult rats in the department of Pharmacology, Katihar Medical College and Hospital, Katihar, Bihar, India. All animal experiments are conducted strictly under the guidelines of the Institutional Animal Ethics Committee. The study protocol was reviewed by the Ethical Committee of the Hospital and granted ethical clearance.

Methodology

The study was carried out with 30 young adult rats weighing between 250 and 300 g, housed in rat cages with ad libitum access to a standard rodent diet and tap water. 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 = 6). 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 = 12). In this group, rats were operated with a method which as described below
- Group 3 (amifostine): Amifostine-treated ischemic kidney group (n = 12). 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 Intervention

Rats were placed on the operation table in the supine position and stabilized on the extremities and they were injected with intramuscular ketamine hydrochloride in a

dose of 35 mg/kg and combined with mild ether anesthesia. 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.
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.

Investigations
Blood Physiochemical Assays:
The whole blood drawn was centrifuged at 4 ^{0}C to obtain the serum sample. The level of blood urea nitrogen (BUN) and serum creatinine (Scr) was measured by the automatic biochemistry analyzer.
Histopathological Investigation
Histopathological investigation was done with regard to the necrosis grading method on rat kidneys described by Jablonski [11]

Statistical Analysis
The recorded data was compiled and entered in a spreadsheet computer program (Microsoft Excel 2010) and then exported to data editor page of SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics included computation of percentages and means. The group level differences of BUN, Creatinine and GSH levels were evaluated with "Kruskal–Wallis test" and for comparing the intergroup mean levels "Mann–Whitney U-test" were used. The results of histopathological examinations were analysed using Chi-square test. The confidence interval and p-value were set at 95% and ≤ 0.05 respectively.

Results

Table 1: Mean Blood Urea Nitrogen Levels among study groups at day 3 and day 7

Blood Urea Nitrogen	Day 3	Day 7	p-value
Group I	31.32	34.34	0.031 (sig.)
Group II	36.21	42.56	0.001 (Sig.)
Group III	35.63	35.79	0.971 (NS)
p-value	0.001 (Sig.)	0.001 (Sig.)	

Table 1: In all three groups, serum mean BUN levels at the 3rd and 7th day were found to be statistically significant. 7th day serum mean BUN levels were found to be the highest in group II (p<0.05) respectively.

Table 2: Mean Serum Creatinine Levels among study groups at day 3 and day 7

Serum Creatinine	Day 3	Day 7	p-value
Group I	0.70	0.59	0.031 (Sig.)
Group II	0.91	0.82	0.029 (Sig.)
Group III	0.81	0.78	0.781 (NS)
p-value	0.024 (Sig.)	0.001 (Sig.)	

Table 2: In all three groups, serum mean creatinine levels at the 3rd and 7th day were found to be statistically significant. 7th day serum mean creatinine levels were found to be the highest in group II (p<0.05) respectively.

Table 3: Mean GSH Levels among study groups at day 7

Groups	Mean GSH
Group I	85.12
Group II	66.0
Group III	105.36
p-value	0.001 (Sig.)

Table 3: Mean GSH levels in kidneys of rats in all three groups on the 7th day were evaluated, levels were statistically significantly different. There was a statistically significant difference between group II and III (p<0.05) respectively.

Table 4: Mean Level of Necrosis recorded among study groups at day 7

Grades of Necrosis	Group I	Group II	Group III
0	6	0	3
1	0	4	6
2	0	2	1
3	0	3	1
4	0	2	0
Total	6	12	12
p-value	0.001 (Sig.)		

Table 4: At 7th day after staining of all kidneys evaluation with respect to necrosis grade yields statistical significant difference (p < 0.05). In the group I, necrosis was not encountered in any of the kidneys at day 7. Grade 1 and 2 necrosis in 6 kidneys, grade 3 and 4 necrosis in the other 5 kidneys was detected in group II. Grade 4, necrosis was not detected in kidneys of amifostine group.

Discussion
Ischemia-reperfusion injury (IRI), which presents in numerous clinical conditions including renal transplantation, partial nephrectomy, shock, cardiac surgery, and vascular surgery, is a major cause of high morbidity, increased Medicare costs, and high mortality [12, 13]. Notwithstanding, there is no particular treatment to treat or forestall to date. Hence, it is a critical need to locate a viable treatment. A preclinical animal model may offer us novel underlying pathophysiological mechanisms and new opportunities for therapeutic intervention in humans
In this study at 3rd and 7th day in all groups, there was an increase in BUN levels, 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. These results indicate that amifostine plays a preventive role on I/R injury. Our results are comparable to those of Valdivielso *et al*. [14] and show that RIR-induced oxidative stress is associated with impaired renal function leading to a marked increase in serum creatinine in non-treated rats.
Spencer and Goa [15] 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). Similarly, in the present study amifostine group GSH levels were increased. This finding indicates that amifostine causes decrease in free radical levels in the kidney.
In the present study at 7th day all kidneys evaluation with respect to necrosis grade yields statistically significant

difference ($p<0.05$). Grade 1 and 2 necrosis in 6 kidneys, grade 3 and 4 necrosis in the other 5 kidneys was detected in group II. 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.

Conclusion

In this study, it was concluded that amifostine can protect tissues from the harmful effects of ischemic reperfusion injury. Amifostine exposes this effect by increasing the level of reduced GSH, which is a well-known oxygen radical eliminator. Further studies should be performed to better elucidate the role of amifostine in ischemic reperfusion injury and to determine whether it can be used clinically to attenuate the damage induced in humans.

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