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Full Length Research Paper

The protective effect of silymarin on the antioxidant system at rat renal ischemia/reperfusion injury model

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The aim of this study is to reveal the protective effects of silymarin (SM) treatment on the generation of oxidative stress with rat renal ischemia/reperfusion (I/R) injury model. Thirty-two (32) Sprague-Dawley rats were evaluated in four groups. Group I (Sham), Group II (renal I/R), Group III (renal I/R injury + SM 100 mg per kg) and Group IV (renal I/R injury + SM 200 mg per kg) were designed to evaluate dose-dependent effects of SM in renal I/R injury on the morphological and biochemical parameters changes. Renal I/R significantly decreased the enzymatic activity of catalase (CAT) and superoxide dismutase (SOD), whereas the malondialdeyde (MDA) levels increased. After renal I/R injury, significant tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury was observed. In the Groups III and IV, in which the rats were treated with SM before renal I/R, tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury were observed to be significantly protected with the treatment. The results of this study have demonstrated that SM significantly prevents the generation of oxidative stress and renal I/R injury induced renal changes in the rat.

Key words: Kidney, oxidative stress, pathology, rat, silymarin, morphology.

INTRODUCTION

Renal ischemia/reperfusion (I/R) injury, which occurs in many clinical during the course such as partial nephrectomy, renal artery angioplasty, trauma, shock, major vascular surgery, sepsis and renal transplantation, is associated with increased mortality and morbidity rates due to acute renal failure (ARF) (Thadhani et al., 1996; Takada et al., 1997; Matin and Novick, 2001; Avlan et al., 2006). Reperfusion of the ischemic tissue may produce reactive oxygen species (ROS), which are known to have deleterious effects such as increased microvascular permeability, interstitial edema, impaired vasoregulation, inflammatory cell infiltration and necrosis (Granger and Korthuis, 1995). In ischemic, ARF leads to a complex cascade of events which are also known to include the activation of nuclear factor kappa B (NF-κB), which controls cytokine, chemokines and adhesion molecules (Rodrigo and Bosco, 2006). Oxidative stress is a relative excess of oxidants caused by increased free radical production and/or decreased antioxidant defense systems that impairs cellular function and contributes to the pathophysiology of many diseases (Karimi et al.,

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2005; Zhao, 2005). The antioxidant defense systems, none enzymatic free radical scavengers (vitamin E, vitamin C, uric acid and billirubin) and the antioxidant scavenging enzymes, [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] protect cells and tissues against oxidative injury (Granger and Korthuis, 1995; Marubayashi and Dohi, 1996; Zhao, 2005). Naturally occurring flavonoids have antioxidant effects due to their phenolic structures and have been reported to inhibit some free radical-mediated processes (Havsteen, 1983; Mora et al., 1990; Zhao, 2005). Silymarin (SM) is a mixture of three isomeric flavonolignans extracted from the milk thistle *Silibum marianum*. SM has been used in most of the remedies for liver disease. Hepatoprotective effects of SM have been attributed to its scavenging ROS, reduction of lipoperoxidation of cell membranes (Farghali et al., 2000). However; very few studies have been performed on oxidative stress with SM in relation to the kidney (Turgut et al., 2008). SM has also been reported to have beneficial effects to protect acute cisplatin nephrotoxicty (Karimi et al., 2005). In our previous study, we demonstrated that SM significantly prevents renal I/R injury induced histopathological changes in the rat kidney (Senturk et al., 2008). This study aimed to re-investigate the possible protective effect of SM against oxidative stress-induced during kidney I/R injury, by determining biochemical parameters and evaluating histological examinations.

MATERIALS AND METHODS

The experimental protocols were approved by the Institutional Animal Ethics Committee. Animals were obtained from Medical and Surgical Experimental Research Center (Eskisehir-TURKEY) and all experiments were carried in same center.

Animals

Thirty-two (32) adult male Sprague-Dawley rats weighting 220 to 250 g were used in the experiment. Rats were housed in polycarbonate cages in a room with controlled temperature (22 ± 2°C), humidity (50 ± 5%), and a 12 h cycle of light and dark; they were fed with laboratory pellet chows and water was given *ad libitum*. The experiment was performed after a stabilization period in the laboratory for 5 days.

Experimental design

Four groups were designed. Group I (Sham) was designed as the control group. Group II (renal I/R) was designed to renal I/R injury. Groups III (renal I/R injury + SM 100 mg per kg) and Group IV (renal I/R injury + SM 200 mg per kg) were designed to evaluate SM on the morphological and biochemical changes in the rats kidney in renal I/R injury.

Right nephrectomies

Right nephrectomies were performed under xylazine (10 mg per kg) and ketamine (70 mg per kg) anesthesia in all rats in all Groups (I to IV). Thereafter, rats were let to recover for 15 days in the standard laboratory.

Drug administration

Seven (7) days prior to I/R induction, 0.5 ml of 100 and 200 mg/kg SM solution (Sigma-Aldrich, S0292-50G, Italy) were administered orally (p.o.) to the rats in Groups III and IV, respectively. Rats in Groups I and II received 0.5 ml normal (0.9%) saline p.o for 7 days prior to sham operation and I/R induction, respectively.

Induction of renal I/R injury

All surgical procedures were performed under xylazine (10 mg per kg) and ketamine (70 mg per kg) anesthesia. Renal I/R injury were induced with left renal pedicle occlusion with a vascular clamp for 45 min followed with reperfusion for 6 h through a median laparotomy under anesthesia. Sham procedures were same beyond vascular occlusion in the Group I. After induction of I/R injury in Groups II, III and IV, left kidneys were dissected for both biochemical and histopathological examinations.

Histopathological evaluation

Left kidneys specimens were processed routinely in 10% formalin solution, and embedded in paraffin. Tissue sections of 5 µm were obtained and stained with hematoxylin and eosin (H&E). Histopathological examinations were performed under a light microscope (NIKON, Japan). All histopathological examinations were performed by the same pathologist of the institute, who was blinded to all the tissue specimens. A minimum of 10 fields for each kidney slide with minimum ×50 magnification were examined to assign the severity of these morphological changes. The morphological changes were scored on a scale of none (−), mild (+), moderate (++) and severe (+++) damage in order to perform a comparison between the groups.

Biochemical analysis

Postmitochondrial supernatant preparation (PMS)

After sacrificing the animals, isolated areas of the nephron of their kidneys were quickly removed and wash immediately with ice-cold normal saline, and homogenized in chilled potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 × *g* for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 × *g* for 20 min at 4°C to get the PMS which was used to assay malondialdehyde (MDA), CAT and SOD activity.

The protocols of lipid peroxidation and enzyme activities measurement

Determination of lipid peroxides (measured as MDA)

MDA, a reactive aldehyde, that is, a measure of lipid peroxidation, was determined according to the method of Uchiyama and Mihara (1978). The adducts formed following the reaction of tissue homogenate with thiobarbituric acid in boiling water bath, were extracted with n-butanol. The difference in optical density developed at two distinct wavelengths, 535 nm and 525 nm was a measure of the tissue MDA content. Tissue MDA content was

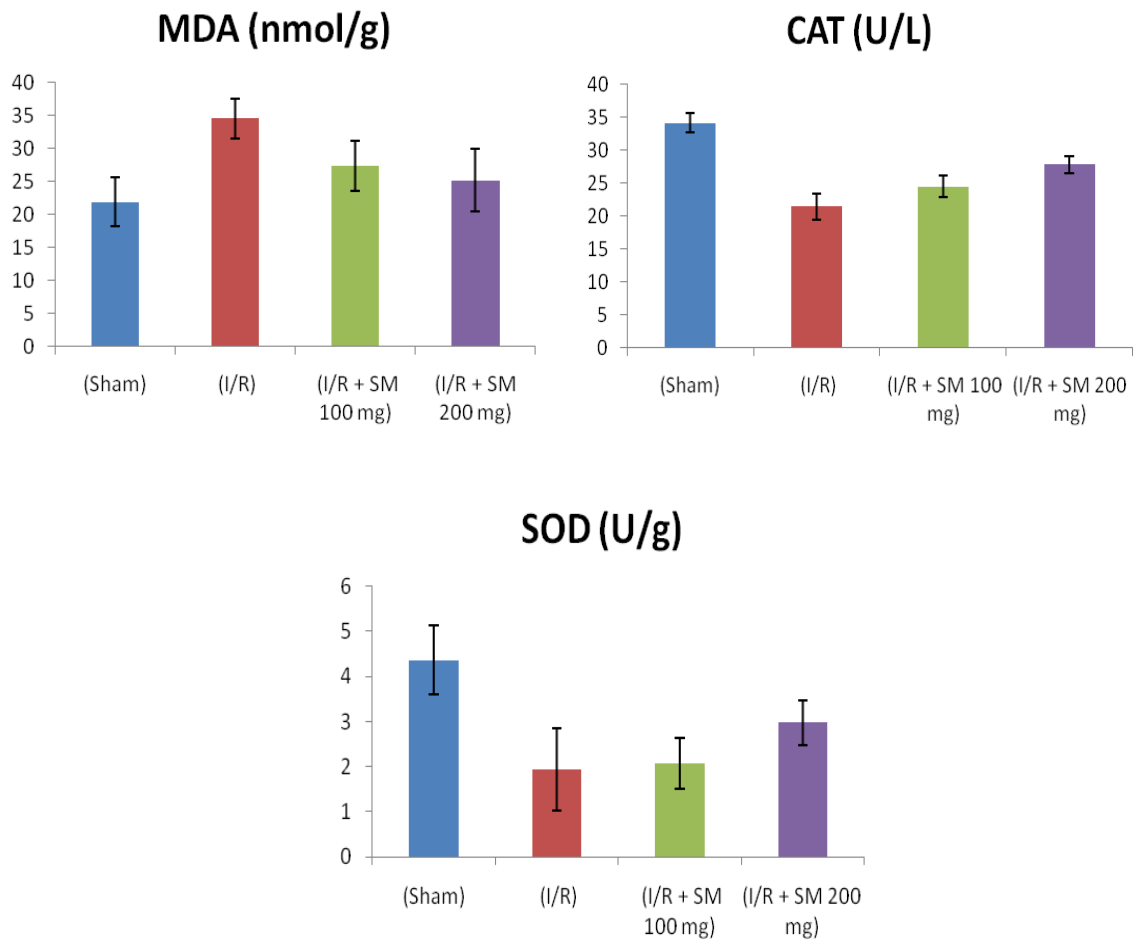

Figure 1. Effect of SM treatment on kidney tissue content of MDA, SOD and CAT. Rats in sham and I/R groups were administered normal saline 7 days prior to I/R induction. Rats in Groups III and IV were administered 100 and 200 mg/kg SM 7 days prior to I/R induction. Data were expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$.

expressed as nmol/g tissue (Mihara and Uchiyama, 1978).

Determination of SOD activity

SOD *activity* was spectrophotometrically assayed with commercial kits. The Fluka SOD kit USA contains all reagents and solutions required for determining SOD activity in an indirect assay method based on xanthine oxidase and a novel color reagent. The homogenate SOD activity was determined by inhibition of Formosan dye (450 nm) employing the xanthin-xanthin oxidase enzymatic method to generate superoxide radicals and expressed as U/g.

Determination of CAT activity

One unit (1 U) of CAT equals the enzyme activity that recognized 1 µmol of hydrogen peroxide in 60 s at 37°C. The three blank samples were prepared according to Goth, 1991. CAT activity was measured with determination of absorbance of three blank samples at 405 nm in spectrophotometer. CAT activity (kU/L) was calculated as = [(Abs_{blank1}- $Abs_{blanksample}$) / ($Abs_{blank\ 2}$- $Abs_{blank\ 3}$)] x 271 (Goth, 1991).

Statistical analysis

All statistical analysis was performed with the computer program "SPSS for Windows'' (SPSS Inc; Release 11.5; Sep 6, 2002). All of the data were expressed as means ± SD. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The significance was tested at $p > 0.05$, $p < 0.05$, $p< 0.01$ and $p < 0.001$.

RESULTS

Renal I/R significantly decreased the enzymatic activity of CAT and SOD, whereas the MDA levels increased. This enzymatic activity levels was significantly improved by treatment with both SM 100 (Group III) and 200 (Group IV) mg (Figure 1).

Light microscopic evaluation revealed that normal renal morphology in the Group I (Sham), and some of the histopathological findings, which were observed in renal I/R injury in Groups II, III and IV (renal I/R; renal I/R injury + SM 100 mg per kg; and renal I/R injury+SM 200 mg per

Table 1. Effect of SM (100 and 200 mg per kg, per oral) treatment on morphological changes as assessed by histopathological examination of kidneys of the rats exposed to renal I/R.

Group	Tubular necrosis	Glomerular necrosis	Tubular dilatation	Necrosis of epithelium	Hyaline casts	Interstitial inflammation
Group I (Sham)	-	-	-	-	-	-
Group II (I/R)	+++	+++	+++	+++	+++	+++
Group III (I/R + SM 100 mg)	-	+/-	+/-	-	+/-	+/-
Group IV (I/R + SM 200 mg)	-	+/-	+/-	-	+/-	+/-

Silymarin, SM; (-), none; (+), mild; (+/-), mild/none; (++), moderate; (+++), severe.

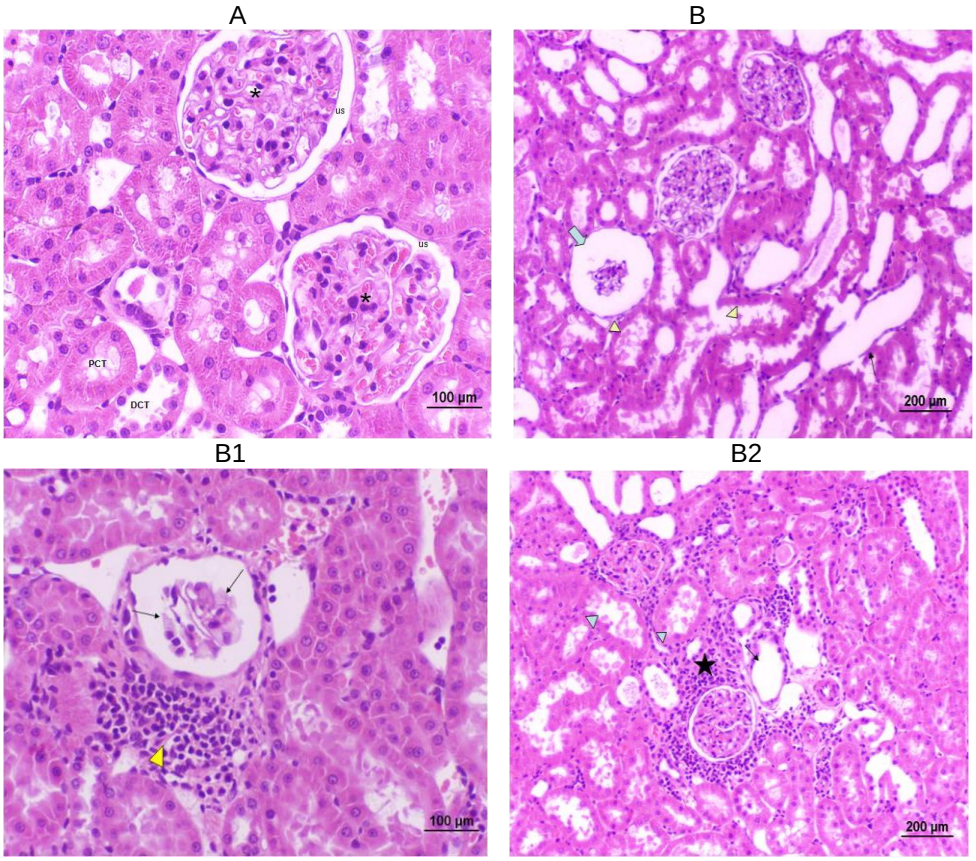

Figure 2. (A), Control group: Renal corpuscle and tubules were observed normal histological structure. (Glomerulus (*), urinary space (US), proximal convoluted tubules (PCT) and distal convoluted tubules (DCT), original magnification × 100. **(B),** Group II (renal I/R); some tubules were desquamation of its epithelial cells (arrow head) and tubular dilatation (thin arrow). Displacement and shrinkage of glomerular tuft is also seen in this figure (thick arrow). **(B1),** Glomerular tuft was observed shrinkage and degeneration (arrow). Inflammatory cells were observed in the intertubular spaces of this figure (arrow head). **(B2),** Some renal tubules were observed desquamation of its epithelial cells (arrow head) and tubular dilatation (arrow). Inflammatory cells were observed in the intertubular spaces of this figure (*).

kg, respectively) (Table 1).

In Group II (renal I/R injury), significant tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium were observed due to renal I/R injury. In the Groups III and IV, in which the rats were treated with SM 100 and 200 mg per kg before renal I/R, tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury were observed to be protected with the treatment (Figures 2 and 3).

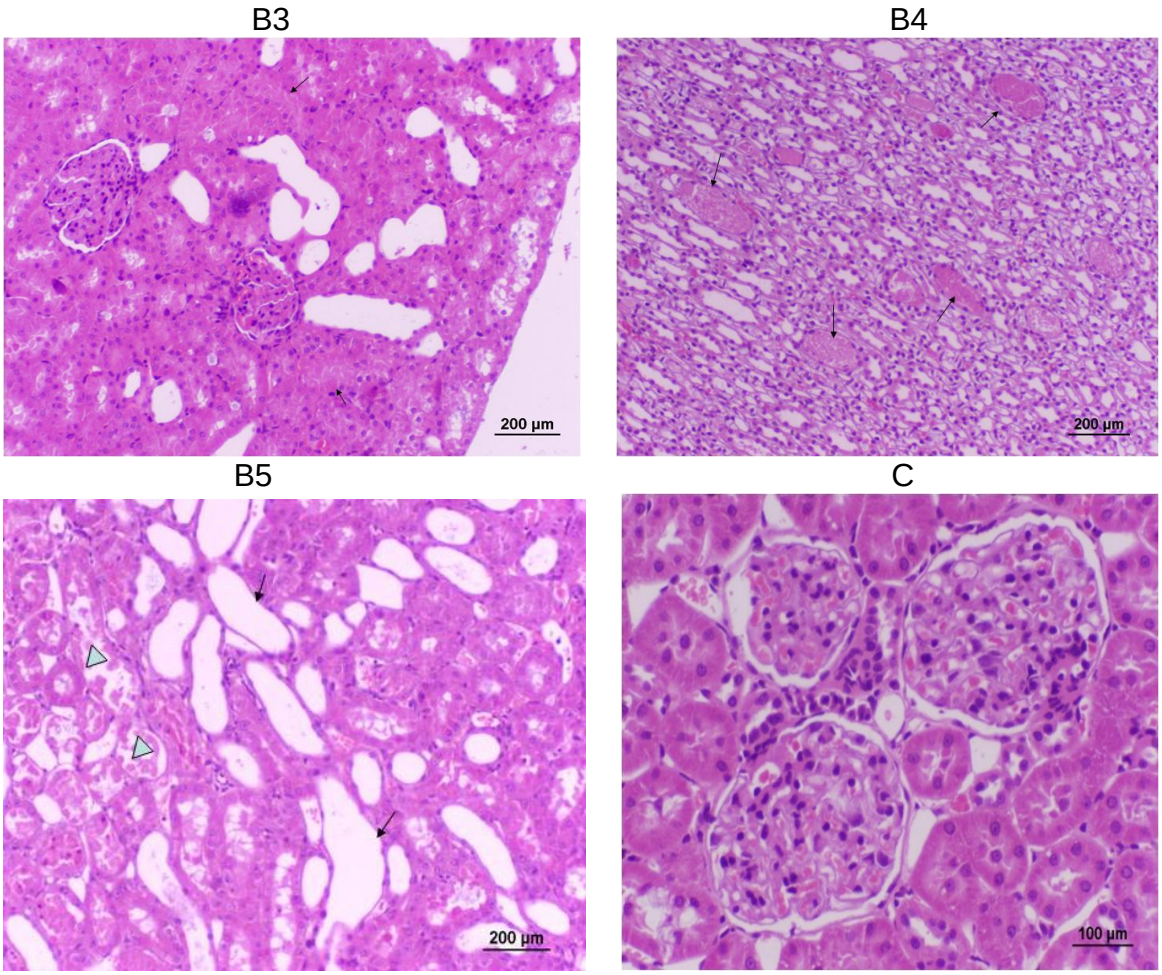

Figure 3. (B3), Widespread tubular necrosis and necrotic cells of the proximal tubules were observed in this figure (arrow). **(B4),** Hyaline casts were observed tubular structure (arrow). **(B5),** Epithelial cells of renal tubules were observed desquamation. Necrotic cells were seen in tubule lumen (arrow head). Also, some tubules were observed tubular dilatation (arrow). **(C),** Kidney section of SM (100 mg per kg, per oral) + renal I/R treated rat showing normal renal corpuscule and tubules.

DISCUSSION

Oxidative stress plays an important role in kidney I/R injury (Granger and Korthuis, 1995). Thus, increasing the kidney antioxidant capacity could be a promising therapeutic approach. Despite improvements in organ preservation and surgical techniques, I/R injury remains a significant clinical problem, and there is considerable interest in its prevention.

Several studies have been reported on the protective effects of antioxidants in different organ and renal I/R injury (Huang et al., 1995; Sehirli et al., 2003; Sener et al., 2004; Sener et al., 2006). Recent approaches advocated to control the production of ROS, which may directly lead to per oxidation of cell membrane lipids and permanent cellular damage, have been generally designed as the therapies including antioxidants such as n-acetylsystein (NAC), revesatrol, vitamin E, and others (Sener et al., 2006; Thurman, 2007).

Tissue ischemia not only leads to the over production of ROS which directly induces tissue damage, but also triggers an aggravated local and systemic inflammatory response that causes multiple organ failure. Several studies demonstrated a recruitment of the neutrophils into post ischemic tissue, but activated neutrophils are also reported to be a potential source of ROS (Zimmerman et al., 1990; Granger and Korthuis, 1995). Chemokines, such as Interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF-α) released from cellular elements, nitric oxide synthase (NOS) which modulates nitric oxide (NO) levels, adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and NF-κB have been recently studied in several organ I/R injury models (Thurman, 2007). Results of these studies suggest that treatment and even protection of tissue and organ damage due to I/R may be possible with modulation of

the elements mentioned previously (Weight et al., 1996; Donnahoo et al., 1999).

The active constituents of milk thistle are flavonolignans including silybin, silydianin and silychristine, collectively known as silymarin. Medical use of milk thistle as a liver protecting herb dates back to the earliest Greek references to the plant. Hepatoprotective effects of SM are mainly attributed to its antioxidant, anti-inflammatory and antifibrotic activity (Ferenci et al., 1989; Luper, 1998). Also, recently, it has been reported that, induction of NF-κB with TNF-α and IL-1β was mediated through intracellular calcium but not ROS. The same report has showed that SM inhibited TNF- α-induced calcium-dependent NF-κB activation irrespective of its antioxidant effect in human mesangial cells (Chang et al., 2006).

These protective effects may be related to different mechanisms such as the scavenger activity of the free radicals that induce lipid per oxidation (LP), and also stimulating antioxidant regeneration through increased protein synthesis (Sonnenbichler and Zetl, 1986). In experimental hepatic injury models, SM was reported to be effective on LP which mainly leads to destruction of plasma membrane (Moscarella et al., 1993; Farghali et al., 2000).

Further study is needed to overcome the limitations of this current study and to verify the significance of the results. The authors believe that the limitation of this study is quantitative measurement of apoptosis and may add some objective supporting data to our results to clarify the effect of SM in renal I/R injury. I/R injury caused an impairment in renal function (increased serum creatinine and blood urea nitrogen (BUN) levels along with significant decrease in creatinine clearance), in our study was not evaluated in this issue.

The protective effects of SM on the primary inflammatory cell, renal tubular epithelium, in renal I/R is thought to be both due to the inhibition of NF-κB and anti-oxidative activity of SM. Further specific study is needed to clarify this issue such as measurement of tissue myeloperoxidase activity (MPO). SM has been reported to be safe to use in various conditions with minimal adverse effects (Jacobs et al., 2002). However, the adverse effects and the safety of SM were not in the scope of our study. The protective effects of SM was observed in even with 100 mg/kg in Group III (SM 100 mg/kg + renal I/R), with increased dose of SM in Group IV (SM 200 mg/kg + renal I/R) prevent the morphological changes in all rats. However, SOD and CAT levels suggested that the higher levels of SM may be more effective in preventing oxidative injury.

Conclusion

The results of our study have demonstrated that SM significantly prevents renal I/R injury-induced renal changes in the rat. The clinical implications of these results merits further experimental and clinical studies to be performed.

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