AMELIORATIVE EFFECTS OF CAPSAICIN ON RENAL DAMAGE INDUCED BY ISCHEMIA/ REPERFUSION INJURY VIA CONTROLLING APOPTOSIS AND ANTIOXIDANT STATUS

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ABSTRACT

Renal ischemia is a principal source of acute renal failure and results in high rates of morbidity and fatality. Several natural products have been reported to have beneficial effects on ischemia/reperfusion (I/R) injury, particularly from a preventative perspective. Therefore, this study was designed to investigate the efficiency of capsaicin, a natural product derived from chili peppers on renal dysfunction and injury induced by I/R in rat kidney. Rats were subjected to renal ischemia by occluding both renal arteries for 60 min followed by 6 h reperfusion to induce renal injury. I/R injury was induced in rats alone or in combination with capsaicin at dose of 1.0 and 3.0 mg/kg with seven days pretreatment. Exposure to I/R injury cause renal function impairment as assessed by measuring blood urea nitrogen and serum creatinine levels. Exposure to I/R injury decreased the activities of glutathione peroxidase and increased glutathione-S-transferase level in the kidneys of rats and increased apoptosis in renal cells as assessed from MTT assay. Capsaicin pretreatment significantly improved renal function, renal mitochondrial antioxidant status and secured cellular viability, thus demonstrating the protective effect in ischemic renal tissue in rats. Supplementation and/or treatment with capsaicin could exert protective effects against renal damage resulting from hypoxic and ischemic injury.

KEYWORDS: apoptosis, capsaicin, ischemia and reperfusion, renal damage.

INTRODUCTION

Oxidative stress is a state of physiological or psychological responses caused by adverse stimuli that tend to disturb the functioning of an organ. Chronic stress increases the oxygen free radicals levels and influences on the function of antioxidant defense system enzymes.[1] One specific and classic method to induce psychological and physiological stresses simultaneously is ischemic condition which alters either the activities of antioxidant enzymes or their capacities in some organs including brain, liver and kidneys.[2] Renal ischemia is a principal source of acute renal failure and results in high rates of morbidity and fatality. Furthermore, acute renal injury is a common health problem with increasing outrageous incidences and still meagre salutary preferences.[3] Postoperative acute renal failure in consequence of ischemia and reperfusion (I/R) injury can occur after kidney transplantation.[4] The pathophysiological mechanisms leading to acute ischemic renal failure are not completely understood. Severe reduction of renal blood flow causes cell damage by high-energy phosphate depletion and the subsequent failure to maintain physiological ion gradients across the cellular membrane. I/R injury is considered as an inflammatory process originally triggered by tissue oxygen starvation, mitochondrial dysfunction and ATP depletion. Upon hypoxic injury, tubular epithelial cells acquire a pro-inflammatory phenotype and start to release cytokines and chemokines.[5] Reperfusion with oxygenated blood is associated with the generation of free radicals and thus lipid peroxidation, polysaccharide de-polymerization and DNA degradation. Injured endothelial cell fail to initiate the relaxation of vascular smooth muscle cells, release potent vasoconstrictors and swell; the permeability is increased and finally, leukocytes and platelets are trapped and accumulate in the microcirculation and the tissue. Eventually this results in a progressive loss of perfusion and further tissue damage.[6] However, there is no clinically effective therapy that prevents ischemic injury completely. Several investigations regarding the implication of reactive oxygen species (ROS) on renal I/R have demonstrated the beneficial effects of pharmacological interventions, such as the prevention of ROS generation, inhibition of enzymes responsible for ROS generation, administration of antioxidant enzymes and scavenging of ROS molecules.[7] Although some pharmacological interventions have provided promising results against

renal I/R injury, the potential benefits of systemic clinical administration of these agents have been limited due to several confounding factors. On the other hand, there is growing interest in natural products as agents to manage health, particularly from a preventative perspective. Several plant-derived agents have been reported to have beneficial effects on renal I/R injury.

Compounds known as capsaicinoids cause the spicy flavor (pungency) of red chili pepper fruit (*Capsicum annuum*) and the primary capsaicinoid in chili pepper is capsaicin. Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) has been extensively studied for its biological effects which are of pharmacological relevance.[8] These include: cardio protective influence, anti-lithogenic effect, anti-inflammatory and analgesia, thermogenic influence and beneficial effects on gastrointestinal system. Therefore, capsaicinoids may have the potential clinical value for pain relief, cancer prevention and weight loss.[9] It has been shown that capsaicinoids are potential agonists of capsaicin receptor (TRPV1).[10] The involvement of neuropeptide substance P, serotonin and somatostatin in the pharmacological actions of capsaicin has been extensively investigated. Topical application of capsaicin is proved to alleviate pain in arthritis, post-operative neuralgia, diabetic neuropathy, psoriasis etc.[11] Capsaicin inhibits acid secretion, stimulates alkali and mucus secretion and particularly gastric mucosal blood flow which helps in prevention and healing of gastric ulcers. Beneficial influences of capsaicin on gastrointestinal system include digestive stimulant action and modulation of intestinal ultrastructure so as to enhance permeability to micronutrients.[12]

So far, there are no findings to prove that treatment with capsaicin could improve the survival rate after renal I/R injury. Hence, the aim of present investigation was to evaluate the potential of capsaicin against renal I/R induced vascular mutilation in rat by assessing renal function and various biochemical parameters.

MATERIALS AND METHODS

Chemicals
Capsaicin (purity: ≥95%) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. All other chemicals and reagents used were of analytical grade. Dose of capsaicin was prepared in vehicle composed of 10% tween 80, 2% ethanol and 0.9% saline solution.[13] All other chemicals were dissolved in double distilled water. The doses for all freshly prepared drug solutions were expressed in terms of their free bases.

Subjects
Sprague-Dawley male rats (250-300 g and 8 weeks or older) were used in this study. Animals were grouped six rats per cage and maintained at standard conditions of humidity (55 ± 5%), temperature 24 ± 2 °C under 12:12 h light/dark cycle. Animals have free access to rodent chow and tap water *ad libitum*. Animal studies were conducted after getting approval from Institutional Animal Ethics Committee.

Treatment schedule
Animals were divided into five groups consisting six animals each.
Group I [Normal Control]: animals of this group were treated with vehicle (p.o.) for seven days.
Group II [Drug Control]: animals of this group were treated with capsaicin (3.0 mg/kg, i.p., twice daily) for seven days.
Group III [Ischemic Control]: animals of this group underwent renal artery occlusion for 60 min followed by 6 h of reperfusion.
Group IV [I/R + C (1)]: animals of this group were pretreated with capsaicin (1.0 mg/kg, i.p., twice daily) for seven days before I/R injury.
Group V [I/R + C (3)]: animals of this group were pretreated with capsaicin (3.0 mg/kg, i.p., twice daily) for seven days before I/R injury.

The dose of capsaicin used in this study was based on previous *in vivo* study indicating the preventive effect of capsaicin on oxidative stress in rats[13] and were selected based on preliminary studies carried out in our laboratory. Acute and chronic toxicity studies and pharmacological studies[13] conducted on capsaicin were used as reference in deriving the current doses.

Experimental induction of I/R
At the end of the treatment period animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (50.0 mg/kg) and xylazine (10.0 mg/kg). The rats were placed on a heating pad kept at 37 °C to maintain constant body temperature. A midline incision was made, the renal pedicle observed and arteries bilaterally occluded with an atraumatic microvascular clamp for 60 min. The time of ischemia was chosen to maximize reproducibility of renal functional impairment while minimizing mortality in these animals. After 60 min of renal ischemia, the clamps were removed and the kidneys were inspected for restoration of blood flow. The abdomen was closed in two layers. Sham-operated animals underwent the same surgical procedure without clamp application. Following 6 h of reperfusion period, animals were sacrificed by cervical dislocation.[14] At the time of death, blood was collected by heart puncture for measurement of biochemical analysis and kidneys were removed.

Assessment of renal function
Collected blood was centrifuged at 7500 rpm for 15 min at °4 C. Then serum was transferred using micropipette in micro-centrifuge tubes and stored at 4 °C till analyzed. Serum samples were assayed for blood urea nitrogen (BUN) and creatinine using standard diagnostics kits.

Assessment of antioxidant status and apoptosis
Post-mitochondrial supernatant and microsome preparation

Tissue processing and preparation of post-mitochondrial supernatant (PMS) were done as described by Athar and Iqbal (1998). Rats were sacrificed, kidneys were removed quickly, cleaned free of extraneous material. The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using homogenizer. The homogenate was filtered through muslin cloth and was centrifuged at 800 g for 5 min at 4 °C to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4 °C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge at 34000 rpm at 4 °C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%). All the biochemical determination were completed within 24 h of animal sacrifice.[15]

Assay for glutathione peroxidase activity
Glutathione peroxidase (GPx) activity was measured by the method of Mohandas *et al.* (1984). The reaction mixture consisted of 1.44 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of 6.22×10^3/M/cm.[16]

Assay for glutathione-S-transferase activity
Glutathione-S-transferase (GST) activity was assayed by the method of Habig (1974). The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml, 1,chloro-2,dinitrobenzene (CDNB) (1 mM) and 0.3 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzyme activity

was calculated as nmol CDNB conjugate formed per minute per mg protein using a molar extinction coefficient of 9.6×10^6/M/cm.[17]

Assessment of apoptosis (cellular viability using MTT assay)
The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reduction was used to assess the activity of the mitochondrial respiratory chain as an indicator for cellular viability. The kidney slices were incubated with MTT (45 µg/ml) for 45 min at 37 °C. Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of the soluble yellow MTT dye to the insoluble purple formazan. The formazan crystals formed were solubilized in iso-propanol and the absorbance was measured at 595 nm and the results were compared to those obtained with control samples.[18]

Statistical analysis
Differences between groups were analyzed using analysis of variance (ANOVA) followed by Tucky's multiple comparisons test. All data points are presented as the treatment groups mean ± standard error of the mean (SEM). Probability values less than 0.05 were considered statistically significant in all the cases.

RESULTS

Assessment of renal function
One way ANOWA followed by post-hoc test revealed that levels of kidney function markers, BUN and serum creatinine were noticeably ($p < 0.0001$) elevated in ischemic group as compared to the normal rats. Conversely, treatment with capsaicin (1.0 and 3.0 mg/kg) showed significant and dose-dependent decrease in BUN ($p < 0.0001$) and serum creatinine ($p < 0.0001$) levels as compared to the ischemic control group (Table 1). Capsaicin alone does not exert any effect on kidney function markers in normal rats.

Table 1: Effect of capsaicin pretreatment on renal I/R induced alteration in blood urea nitrogen and serum creatinine levels.

Groups	Treatment	BUN (mg/dl)	Serum creatinine (mg/dl)
Normal Control	Vehicle (p.o., 7 d)	18.21 ± 1.04	1.29 ± 0.09
Drug Control	Capsaicin (3.0 mg/kg, i.p., twice daily)	20.82 ± 2.04	1.33 ± 0.12
Ischemic Control	Ischemia followed by reperfusion	83.72 ± 0.80*	6.83 ± 0.45*
I/R + C (1)	Capsaicin (1.0 mg/kg, i.p., twice daily, 7 d) followed by I/R	49.68 ± 0.45#	3.59 ± 0.30#
I/R + C (3)	Capsaicin (3.0 mg/kg, i.p., twice daily, 7 d) followed by I/R	28.97 ± 0.23#	2.36 ± 0.18#

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; #p< 0.0001 when compared with ischemic control group. I/R: ischemia followed by reperfusion; C: capsaicin.

Assessment of antioxidant status and mitochondrial integrity
There was a significant drop ($p < 0.0001$) in levels of GPx and significant increase ($p < 0.0001$) in GST levels in renal tissue of rats induced with I/R injury as compared to normal control group. Chronic treatment with capsaicin (1.0 and 3.0 mg/kg) significantly

increased (p< 0.0001) GPx level and significantly decreased GST level (p< 0.0001) in renal tissue compared to ischemic control group (Table 2). Capsaicin alone does not exert any effect on GPx and GST levels in normal rats.
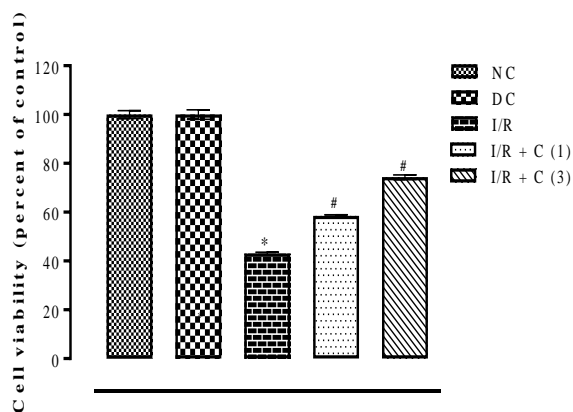
Table 2. Effect of capsaicin pretreatment on renal I/R induced alteration in glutathione peroxidase and glutathione-S-transferase enzymes.

Groups	Treatment	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione-S transferase (nmol CDNB conjugate formed /min/mg protein)
Normal Control	Vehicle (p.o., 7 d)	78.59 ± 0.62	211.35 ± 2.07
Drug Control	Capsaicin (3.0 mg/kg, i.p., twice daily)	82.11 ± 0.79	219.53 ± 2.66
Ischemic Control	Ischemia followed by reperfusion	34.23 ± 0.31*	587.19 ± 4.72*
I/R + C (1)	Capsaicin (1.0 mg/kg, i.p., twice daily, 7 d) followed by I/R	61.95 ± 0.58#	321.84 ± 3.65#
I/R + C (3)	Capsaicin (3.0 mg/kg, i.p., twice daily, 7 d) followed by I/R	86.50 ± 0.78#	239.76 ± 1.99#

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; #p< 0.0001 when compared with ischemic control group. I/R: ischemia followed by reperfusion; C: capsaicin.

Assessment of apoptosis (cellular viability using MTT assay)
The exposure of renal tissue to I/R injury resulted in marked changes in cellular viability in terms of decreased MTT reduction in kidney of rats. Capsaicin (1.0 and 3.0 mg/kg) significantly (p< 0.0001) increased MTT reduction in renal tissue of rats with I/R compared to the respective control group. Capsaicin alone does not have any effect on cellular viability in normal rats as revealed from MTT assay (Figure 1).

Figure 1: Effect of capsaicin pretreatment on cellular viability using MTT assay in renal tissue of rats caused by I/R induced injury.

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; #p< 0.0001 when compared with ischemic control group. NC: normal control group; DC: drug control group; I/R: ischemia followed by reperfusion; C: capsaicin.

DISCUSSION
The present study revealed that renal artery occlusion for 60 min followed by 6 h of reperfusion significantly caused renal damage as assessed by monitoring renal function loss and various biochemical parameters. I/R induced renal injury increased the levels of kidney function markers, BUN and serum creatinine. I/R induced renal damage also significantly decreased GPx level and increased GST level in renal tissue. In addition, marked changes in cellular viability in terms of decreased MTT reduction in kidney of rats was observed as a measure of cellular apoptosis. Pretreatment with capsaicin restored all the impaired conditions.

I/R have been implicated in the pathogenesis of renal injury by directly affecting kidney cells due to an assortment of facets like hypoperfusion, hypoxia, inflammatory reactions and free radical-induced changes.[19] Ischemic renal injury is considered by intra-renal vasoconstriction, leading to reduced glomerular plasma flow and filtration rate and reduced oxygen deliverance to the tubules of the outer medulla. Moreover, free radicals cause DNA scission and base modification, lipid peroxidation, protein damage and inactivation by their chemical modification, leading to cell death.[20] Thus selected animal model of I/R produced severe impairment in renal function.

A rapid change in serum creatinine is the largely frequent sign of acute kidney injury. Due to excess of creatinine, acute inflammatory edema and tubular necrosis formation are accompanied by significant changes in the incidence of cellular proliferation.[21] There are ample of reports concerning decrease in glomerular filtration rate in I/R induced damage in rats because of remarkable elevate in serum creatinine and blood urea nitrogen

levels, that have been reveled in present study also and which are in accordance the earlier findings.[22]

The activation of oxidative stress mediated mitochondrial dysfunction and damage is primarily catalyzed by phase I enzymes, protection may be accomplished by inhibition of activating enzymes and/or by induction of phase II enzymes which leads to protection and accelerated excretion of ROS.[23] Hence, both phase 1 enzyme (GPx) and phase II enzyme (GST) have been considered in present study.

Impairment in mitochondrial function as a result from oxidative stress commences apoptotic cascade and conclude in cellular damage. Secondary cell death during apoptosis in ischemia is originated through releasing numerous apoptogenic factors such as apoptosis inducing factor, cytochrome C and caspases.[24] In our study mitochondrial apoptosis has been examined by investigating cell viability by MTT assay. Prohibition of renal cell death by capsaicin resulted in outcome of more viable cells as evident from MTT assay.

CONCLUSION

In conclusion, capsaicin amelioration of I/R induced renal dysfunction is attributed to the enhancement in antioxidant defense mechanism and improvement in the apoptotic procedure. Thus, supplementation and/or treatment with capsaicin could exert protective effects against renal damage resulting from hypoxic and ischemic injury. However, further studies are required in order to gain more insight at molecular level.

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