The Beneficial Effect of Korean Red Ginseng on Rat Bladder Contractility and Oxidant Damage Following Ischemia/Reperfusion

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Purpose: N-acetylcysteine (NAC) is a potent antioxidant, and a free radical scavenger. We investigated the possible effects of NAC after ischemia/reperfusion (I/R) of rat bladder.

Materials and Methods: I/R injury was induced by abdominal aorta clamping and ischemia for 60 minutes, followed by 120 minutes reperfusion. Twenty rats were divided into four groups: sham operation + saline group (S+S), sham operation + NAC group (S+NAC), I/R + saline group (I/R+S), I/R + NAC group (I/R+NAC). Blood levels of reactive oxygen species (ROS) were determined using the free oxygen radical tests (FORT). Superoxide generation was measured based on lucigenin-enhanced chemiluminescence. The level of malondialdehyde (MDA) was analyzed in order to measure lipid peroxidation.

Results: In I/R+S group, the isometric contractile responses to carbachol were significant lower than other groups and were reversed by the pretreatment with NAC. The level of FORT and MDA showed a marked increase in I/R+S group compared with S+S group. NADPH-stimulated superoxide production was also significantly increased. I/R+NAC decreased these parameters compared with I/R+S group.

Conclusion: Our results suggest that treatment with NAC reversed the low contractile responses of rat bladder and prevented oxidative stress following I/R. (J Korean Continence Soc 2009;13:142-51)

Key Words: Bladder, Ischemia, Reperfusion, N-acetylcysteine

Introduction

Ischemia/reperfusion (I/R) plays an important role in the etiology of contractile dysfunction of the bladder when induced by partial bladder outlet obstruction in animal models, and in obstructive dysfunction in men [1]. I/R is also a major etiological factor in bladder dysfunction due to aging, atherosclerosis, hypercholesterolemia, and diabetes [2]. As a result of a reduction in blood flow, ischemia can produce bladder dysfunction, such as a decrease in bladder contraction, compliance and capacity with increased post-residual volume [3-4]. Ischemia is not an isolated event and when reversible, it
is followed by reperfusion. Reperfusion may cause a more severe injury than ischemia alone [5]. Reperfusion and re-oxygenation generate reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals, mediated oxidation of biomolecules probably causes partial denervation of the bladder wall with the subsequent development of supersensitivity and bladder instability [6]. Thus, free radical reduction for the treatment of I/R injury may be beneficial for bladder dysfunction secondary to BPH/BOO.

*Panax ginseng* C. A. Mayer has been used for thousands of years in Asia due to its wide spectrum of medicinal effects. It has a wide range of pharmacological and physiological actions, such as antiaging, immunoenhancement, antistress and antitumor [7-9]. For a long time, it was well known that Panax ginseng had protective properties against free radical attack [10-11]. Among the several kinds of Panax ginseng products, Korean red ginseng (*KRG, Ginseng Radix Rubra*) has the most potent multiple pharmacological actions for anticancer, antihypertension, antidiabetes, antinociception, and improving weak body conditions as tonics [12]. Red ginseng protected smokers from oxidative damage and reduced cancer risk associated with smoking [13]. Red ginseng aqueous extract had free radical scavenging activity [14]. Korean red ginseng components also increased the antioxidant enzymes activity in the liver [11]. Although KRG has been investigated for multiple purposes, its effect on I/R injury of the bladder has not yet been elucidated. The present study was performed to determine whether KRG protected the rat bladder against dysfunction and oxidative stress induced by I/R injury.

**Materials and Methods**

1. Administration of KRG

Steamed ginseng (KRG, Cheong-Kwan-Jang) was purchased from the Korea Ginseng Corporation (Dae Jeon, Korea) and was dissolved in distilled water. KRG (100 mg/kg per day) was given orally for 6 weeks before surgery and continued until the end of the experiment.

2. Animal model

Twenty 6-week-old male Sprague-Dawley rats (120±20gm) were divided into four groups: sham operated group (S), sham operated pretreated KRG group (S+KRG), ischemia/reperfusion group (I/R), ischemia reperfusion and pretreated KRG group (I/R+KRG). All animal experiments followed a protocol approved by ethics committee on animal research at Chungnam National University Hospital. The rats were housed in wire-bottomed cages and were acclimatized at 21°C on 12:12-h light-dark cycle and were allowed access to water and chow ad libitum. The S and I/R group were fed tap water while S+KRG and I/R+KRG group fed the water with KRG for 6 weeks. Rats were fasted for 6 hours before the experiments but were provided with tap water or KRG solution. In all protocols a combination of 80 mg/kg Ketamine and 12 mg/kg xylazine was used to induce anesthesia. Throughout the anesthesia, body temperature was maintained using a heating pad. The S and S+KRG group consisted of surgical shams that low abdominal midline incision was made and the abdominal aorta was exposed but left intact under sterile condition. Ischemia was created by clamping the abdominal aorta just above its bifurcation with a vascular clamp (Sugita standard aneurysm clip, holding force of 140g) for 1 hour. Reperfusion was achieved by removing the clamp; reperfusion lasted for 1 day. And then the rats were anesthetized, the bladder of these groups was excised. The bladder dome and body were separated from the bladder base at the level of the ureteral orifices. The bladder was dissected free of fat tissue and blood vessels. The ventral half was divided into longitudinal strips. They were immediately placed in organ baths. The dorsal half was stored at -70°C for biochemical assays.
3. Measurement of blood flow in the urinary bladder

Blood flow in the rat bladder was measured with a laser Doppler flowmeter (Trasonic Systems, USA). Under anesthesia, the laser doppler probe was attached to the serosal side of the bladder and was rigidly affixed. It was used to measure blood flow throughout the experiment. To evaluate the KRG on the blood flow, the mean levels of blood flow of the sham-operated groups were measured. The effect of KRG during I/R operation was also evaluated. In I/R and I/R+KRG group, the mean levels of blood flow during ischemia and reperfusion were evaluated to confirm the I/R operation correctly.

4. Measurement of contractile response

After the bladders were removed and weighed, longitudinal strips were obtained. The bladder strips included muscle and eliminated mucosa. Each muscle strip was mounted in organ baths containing 50ml of Krebs-Henseleit solution (NaCl 99.01mM, KCl 4.69mM, CaCl₂ 1.89mM, MgSO₄ 1.2mM, K₂HPO₄3H₂O 1.03mM, NaHCO₃ 25mM, Glucose 11.1mM). The solution in the baths was aerated with a mixture of 95% oxygen and 5% carbon dioxide and maintained at 37°C. An initial resting tension of 1g was applied for 60 minutes and the responses were recorded isometrically using a force-displacement transducer. A series of three washes, at 15-minute intervals, with Krebs-Henseleit solution followed each of the pharmacological stimulations. The contractile response to carbachol was expressed as force per 100mg bladder muscle. The contractile responses to carbachol (10⁻⁸-10⁻³mol/L) were obtained cumulatively. Contractile responses of the same muscle strips to 120mM KCl were also monitored.

5. Malondialdehyde (MDA) Quantitation

The extent of lipid peroxidation was measured by the amount of MDA, which was assayed by the thio-barbituric acid reactive substances (TBARS) kit (ZeptoMetrix). Reassumed tissue at 50 mg/ml in normal saline, bladder tissue disrupted in a homogenizer and sonicate for 15 second at 40V setting over ice. Homogenize in isotonic media appropriate for sub cellular fraction to study TBARS in plasma membranes, nuclear membranes or organelles. Quantitation of total protein was performed using Micro BCA Protein Assay (Pierce, Rockford, IL, USA). The amount of MDA in 40ul of cell homogenates was measured by a spectroscopic method described in manufacturer's protocol. The data were normalized and presented as nmol per milligram protein MDA generated per 60 minutes of incubation. The absorbance was read by SAFIRE plate reader, using Xfluor4 software (Tecan) at 532nm.

6. Measurement of xanthine oxidase activity

The Amplex® Red assay kit (Molecular Probes, Eugene, OR) was used to detect xanthine oxidase activity. The assay was carried out according to the manufacturer's protocol. Bladder tissue that disrupted in a homogenizer and sonicate were incubated in the standard incubation medium supplemented with 50uM Amplex Red reagent plus horseradish peroxidase (0.1 U/ml). In the reaction mixture, the superoxide spontaneously degrades to hydrogen peroxide (H₂O₂), and the H₂O₂, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin fluorescence was monitored by Luminescence spectrometer (excitation at 560 nm, emission at 590 nm). H₂O₂ concentration was calculated by using a standard curve and was normalized to cellular protein as measured by the Bradford assay.
7. Measurement of NADPH-driven superoxide production

Lucigenin-enhanced chemiluminescence was used to measure superoxide levels. Lucigenin (bis-N methyl-acridinium nitrate) luminescence specifically in the presence of superoxide. Dark-adapted lucigenin solution (5 µmol/L) was prepared in aerated Krebs-HEPES buffer (NaCl 100 mmol/L, KCl 4.7 mmol/L, CaCl₂ 1.9 mmol/L, MgSO₄ 1.2 mmol/L, K₂HPO₄ 1.03mmol/L, NaHCO₃25 mmol/L, Na-HEPES 20 mmol/L, pH 7.4). The bladder strips was transferred into scintillation vials containing Krebs-HEPES buffer with 5uM lucigenin. The chemiluminescence, which occurred over the ensuing 2 minutes in response to the addition of 100uM NADPH, was recorded. The emitted light units, after subtracting a blank, were used as a measure of superoxide production. Values are expressed as a relative light unit (RLU) per 1ug tissue.

8. Statistical Analysis

Data are expressed as the mean ± standard deviation. The SPSS version 12.0 for Windows was used to evaluate the data. One-way and two-way ANOVAs were used for evaluation of contractile responses. For the analysis of other parameters, the Mann-Whitney method analysis of variance was used. Values of p<0.05 were considered statistically significant.

Results

1. Blood flow in rat urinary bladder

Effect of KRG on blood flow in resting state

The mean level of the blood flow of the S group was 36.4±6.9 ml/mim per 100mg tissue and for the S+KRG group, 39.1±9.3 ml/mim per 100mg tissue. The mean level of blood flow in the KRG treated sham operated group was not different compared to that of the untreated sham operated group.

Effect of KRG on blood flow in I/R state

Clamping the abdominal aorta decreased blood flow to the rat bladder 5.2% of the basal level measured before clamping with administration of KRG. Clamping the abdominal aorta also decreased blood flow 5.5% of the basal level measured before clamping without administration of KRG. After removing the clip, the blood flow recovered to the basal level within 5 minutes. When the rats were treated with KRG, the mean levels of blood flow to the bladder during reperfusion were slightly, but not significantly, increased when compared to those not receiving KRG treatment (Table 1).

2. Results of contractile response

Contractile responses of the bladder dome strips of each group to carbachol were determined (Fig. 1). For the 10⁻⁸, 10⁻⁷, 10⁻³M carbachol concentrations, no differences were found for any of the experimental groups. For the 10⁻⁶⁻¹0⁻⁴M carbachol concentrations, I/R caused significant differences between the study groups by means of contractile response to carbachol. Contractile responses in S+KRG group were not different compared to those of the untreated sham-operated group. In I/R group, the contractile responses of the strips to carbachol decreased significantly when compared to I/R+KRG groups. KRG pretreatment restored the contractile response to carbachol (10⁻⁶⁻¹0⁻⁴M) in I/R group.

Contractile responses induced by 120mM KCl were not significantly different between the S group and S+KRG group (19.6±5.1 vs. 15.6±3.3 gram tension/100 mg tissue). The contractility response of I/R (8.5±2.8 gram tension/100 mg tissue) was significantly lower compared with the sham operated groups (S group vs I/R group, p=0.012, S+KRG group vs. I/R group, p=0.025). Contractility to 120mM KCl of bladder domes in rats with KRG (11.9±3.1 gram tension/100 mg tissue) recov-
Table 1. Blood flow in rat urinary bladder

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal level</th>
<th>During ischemia (% to basal)</th>
<th>During Reperfusion (% to basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R</td>
<td>38.5±7.8</td>
<td>2.1±0.6 (5.5±1.6)</td>
<td>35.0±6.5 (90.8±16.9)</td>
</tr>
<tr>
<td>I/R+KRG</td>
<td>34.7±8.8</td>
<td>1.8±0.4 (5.2±1.1)</td>
<td>32.2±6.9 (92.8±19.9)</td>
</tr>
</tbody>
</table>

Numbers are ml/min per 100mg tissue (Tissue perfusion unit)
I/R ischemia reperfusion group, I/R+KRG ischemia reperfusion and pretreated Korean red ginseng group

Fig. 1. Concentration-contraction curves obtained by cumulative addition of carbachol (CCh) to bladder strips. Each point represents the mean±SEM of 5 bladder strips. *indicates I/R+KRG group is significantly different from I/R group (p<0.05, **p<0.01). + indicates I/R group is significantly different from sham operated groups (p<0.01). Values are expressed as gram tension per 100mg wet tissue. S sham operated group, S+KRG sham operated plus pretreated KRG group, I/R ischemia reperfusion group, I/R+KRG ischemia reperfusion and pretreated KRG group.

Fig. 2. Malondialdehyde (MDA) levels in bladder tissue. Bars indicate mean±SEM of 5 rats. Values are expressed as nmol/mg protein. *indicates I/R group is significantly different from sham operated groups (p<0.001). **indicates I/R+KRG group is significantly different vs. I/R group (p<0.05).

3. MDA Quantitation

The mean level of MDA, which is a major degradation product of lipid peroxidation, in bladder samples showed a marked increase following I/R compared with sham operated group (1.42±0.31 vs. 0.44±0.13 nmol/mg protein, p=0.001, Fig. 2). The mean level of MDA in the KRG-treated sham operated group was not different compared to that of the untreated sham operated group (0.37±0.09 vs. 0.44±0.13 nmol/mg protein, p=1.00). The basal level of MDA significantly decreased in I/R+KRG group compared with I/R group (0.75±0.21 vs. 1.42±0.31 nmol/mg protein, p=0.018). KRG treatment reversed lipid peroxidation degradation.

4. Results of xanthine oxidase activity

The mean level of xanthine oxidase activity showed a marked increase following I/R group compared with S
group (2.14±0.56 vs. 1.12±0.27, p=0.008, Fig. 3). KRG administration abolished the increase in bladder xanthine oxidase level (1.46±0.26, p=0.22).

5. Results of NADPH-driven superoxide production

The basal bladder superoxide level of I/R group was highest, but not significantly different from other groups. NADPH-driven superoxide production was increased in the I/R group (5319±291 RLU/1ug tissue for I/R group vs. 3464±688 RLU/1ug tissue for S group and 3363±345 RLU/1ug tissue for S+KRG group RLU/1ug tissue, p<0.05, Fig. 4). NADPH-driven superoxide level was significantly decreased by treatment with KRG (3642±549 RLU/1ug tissue, p=0.03, Fig. 4).

Discussion

I/R plays an important role in the etiology of contractile dysfunction of the bladder when induced by partial bladder outlet obstruction in animal models, and in obstructive dysfunction in men [1]. Partial outlet obstruction induces a decrease in bladder blood flow (ischemia phase) with a resulting decrease in oxygen tension (hypoxia) in the thick bladder wall, followed by an increase in blood flow and oxygen tension after micturition (reperfusion phase) [15]. Bladder overdistension occurs in patients with acute urinary retention secondary to bladder outlet obstruction [16]. Overdistension, as a physiological or pathological stress, has been shown to result in contractile and metabolic dysfunction of bladder [17]. Prolonged overdistension can result in injury to the neural pathways responsible for micturition [18], reduce bladder elasticity, alter the biochemical and neuronal responsiveness of the bladder [16], and subsequently lead to micturition problems. After overdistension, catheterization/decompression induces reperfusion injury in the bladder and that ROS are one of the main contributing factors in this injury [15]. ROS consists of molecular oxygen and all of its aerobic cellular metabolites including superoxide (O$_2^-$) and hydroxyl radical (OH$^-$). ROS that cause lipid peroxidation of cellular membranes, further dysregulating Ca$^{2+}$ homeostasis and perpetuating cell and subcellular organelle membrane damage [19-20]. Studies in an in vitro model of ischemia/re-oxygenation
have demonstrated that the rate and magnitude of bladder contractile failure are directly related to the extracellular calcium concentration, which in turn is directly related to the level of lipid peroxidation after re-oxygenation [21].

A strong association between cellular and subcellular membrane damage, and the contractile dysfunction was found in obstructed rabbit bladders and in men with obstructive dysfunction [22-23]. The reduction in contractility of bladder muscles may be attributed to the impairment in signal transduction originating from the stimulation of muscarinic receptors by carbachol [24]. Another possibility is that ROS may have a role of direct damage to the contractile apparatus in the smooth muscles of the bladder. We demonstrated that I/R caused significant decreases in contractile responses to carbachol in rat bladder dome. Many authors have reported that I/R play an important role in the etiology of contractile dysfunction of the bladder. In this report, we confirmed ischemia/re-oxygenation cause bladder contractile failure by measurement of contractile response to carbachol. And KRG restored the contractile response to carbachol (10^{-6}-10^{-4}M) in I/R group.

In rats, KCl has a neurogenic stimulation effect in addition to the one it has on muscle stimulation by direct depolarization [25]. In this experiment we observed a significant decrease in the contractile response to KCl in I/R groups. This finding means I/R injury results in the neuronal damage of the bladder. We observed that the responses to KCl were affected, but not significantly, by KRG. Our findings have demonstrated that KRG doesn’t ameliorate nerve injury. The present study is limited in that we didn’t perform the contraction in response to electrical stimulation. Hence, the study may not properly indicate neurogenic stimulation result outcomes. Additional studies are needed to confirm the benefits of KRG to the neuronal damage.

Perfusion following the relief of acute bladder distention was associated with a time dependent increase in lipid peroxidation. Increases in lipid peroxidation can lead to nerve and smooth muscle membrane damage [21]. Ohnishi et al [4] noted in rabbits that in vitro ischemia with subsequent re-oxygenation and substrate replacement resulted in a significantly increased rate of MDA generation, a marker for the degree of lipid peroxidation. The MDA concentration in the bladder was significantly increased in I/R group. Treatment with KRG significantly decreased MDA production by ischemia-reperfusion, and interestingly, the MDA concentration in I/R+KRG group was significantly lower than that of I/R group. These data suggest that KRG has an effect to reduce ROS production in the ischemia-reperfusion organs. As increases in lipid peroxidation can produce nerve and smooth muscle membrane damage, KRG may associate with defensive mechanism that reduces lipid peroxidation.

There are various sources of superoxide such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, XO, lipoygenase, mitochondrial oxidase, and nitric oxide synthase. The two major ROS generating systems are the NADPH oxidase and the XO systems [26]. NADPH oxidase catalyzes the reduction of oxygen through electron donation from either NADH (predominantly) or NADPH to generate superoxide (O_2^-). This system is thought to be the predominant driver of O_2^- formation [27]. KRG inhibits the basal superoxide production and NADPH oxidase in the bladder. KRG inhibits generation of ROS that cause damage the cellular membranes. Pretreatment of KRG protects this continuing membrane damage underlies the progressive bladder dysfunction.

XO is an interconvertible form of xanthine oxidoreductase (XOR); the other form isxanthine hydrogenase (XDH). In normal tissue, XO exists as XDH. During ischemia, XDH is cleaved by a calcium-dependent protease that converts it to XO [28]. At the same time, cellular adenosine triphosphate (ATP) is catabolized to hypoxanthine, which accumulates in ischemic tissue. After reperfusion, XO oxidizes hypoxanthine to xanthine, and further oxidizes xanthine to produce uric acid, O_2^-, and hydrogen peroxide [28-29]. A variety of pathophysiological conditions, including hepatic I/R and hemorrhagic
shock, are reported to induce the systemic release of XO [30-31]. In the pathophysiology of bladder I/R, the release of XO might be induced by reperfusion. There is a positive feedback loop in the O$_2^−$ generation associated with XO. KRG itself has ROS scavenging effects, so that KRG might suppress the positive feedback loop of O$_2^−$ generation due to both inhibitions of XO and the ROS scavenging effect.

Many substances have been suggested to be important in the prevention of bladder dysfunction secondary to ischemia and reperfusion. The potential benefits of antioxidants were shown in a study of the effect of vitamin E on partially obstructed rabbit bladder [32]. William have reported the protective effects of L-NAME on rat bladder subjected to an ischemia-reperfusion model [33]. Free radical damage is important in obstructive and ischemic injury comes from studies showing that natural products high in antioxidants protect the bladder against both functional and biochemical damage induced by partial BOO and ischemia [32-33]. Among the product of Panax ginseng, KRG has been shown to exhibit a variety of antioxidative actions [12,34]. KRG is a useful adjuvant for the protection of I/R induced oxidative injury [34]. This indicates that KRG also has protective effects against I/R induced oxidative injury of the bladder. With these experimental results, we can confirm that KRG protects the bladder from I/R injury.

This paper is the first report to evaluate the effect of KRG, a potent antioxidant, on the bladder function and formation of ROS in rat bladder due to I/R injury. KRG pretreatment prevents bladder contractility via decrease production of NADPH oxidase, XO. Here, we suggest that KRG may have a role on the protection of I/R induced oxidant injury in the rat bladder. Furthermore, in vivo and in vitro studies are required to determine whether KRG has an exact protective role in the I/R injury of bladder. Additional studies are needed to confirm the benefits of KRG to the neuronal damage.

## Conclusion

We have demonstrated that I/R leads to bladder dysfunction, as well as the induction of ROS. Bladder dysfunction and the induction of superoxide production were prevented by administration of KRG. KRG may have a therapeutic benefit in patients with acute and chronic urinary retention.

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