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Protective effects of chitosan oligosaccharide on paraquat-induced nephrotoxicity in rats

Sang Pil Yoon [a,b,*], Min Seok Han [c], Jung Woo Kim [d], In Youb Chang [a,e], Hyun Lee Kim [f], Jong Hoon Chung [f], Byung Chul Shin [f,*]

[a] Korean DNA Repair Research Center, Chosun University, Gwangju, Republic of Korea
[b] Department of Anatomy, School of Medicine, Jeju National University, Jeju-Do, Republic of Korea
[c] Department of Internal Medicine, Seonam University, Namwon, Jeollabuk-Do, Republic of Korea
[d] Department of Anatomy, Seonam University, Namwon, Jeollabuk-Do, Republic of Korea
[e] Department of Anatomy, College of Medicine, Chosun University, Gwangju, Republic of Korea
[f] Department of Internal Medicine, College of Medicine, Chosun University, Gwangju 501-759, Republic of Korea

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ABSTRACT

We investigated the apoptotic pathway during paraquat (PQ)-induced nephrotoxicity as well as renoprotective effects of chitosan oligosaccharide (COS) in male Sprague–Dawley rats because increasing evidences suggest that antioxidants may prevent PQ-induced apoptosis. Animals were pretreated with normal saline or COS (500 mg/kg, p.o.) 3 days before PQ (60 mg/kg, i.p.) administration, and were sacrificed at scheduled times after PQ administration. Rats administered PQ showed increased serum PQ concentrations, blood urea nitrogen, and creatinine levels in a time-dependent manner and creatinine clearance was decreased compared with control rats. All of these parameters were reversed significantly by COS pretreatment. After the PQ injection, cell deaths occurred in the proximal renal tubules with increased APE, PUMA, and cleaved caspase-3 expression, while APE and cleaved caspase-3 were immunolocalized mainly in the proximal tubules of control kidneys. COS-pretreated rat kidneys showed increased expression of the above parameters before PQ injection. APE and cleaved caspase-3 were immunolocalized ubiquitously in the renal cortex of COS-pretreated rats until 24 h after the PQ injection. These results showed that PQ-induced nephrotoxicity may be caused by apoptosis in rat kidneys and that COS could protect kidneys from PQ-induced toxicity in association with the basal higher level of APE.

1. Introduction

Much information is available regarding the toxic effects of paraquat (PQ; 1,1′-dimethyl-4,4′-bipyridinium dichloride) on the lung, but less is known about its renal toxicity. After oral administration, PQ is rapidly distributed to various tissues and is found in high concentrations in the kidney (Smith, 1987). Before the onset of acute renal failure, renal clearance of PQ exceeds that of the glomerular filtration rate, indicating that PQ is actively secreted by the proximal renal tubules (Chan et al., 1997; Molck and Friis, 1998). PQ is also nephrotoxic in the proximal convoluted tubules as a result of high concentrations (Prashad et al., 1981). Patients with PQ intoxication usually require more than 20 h arriving to hospital (Huoze et al., 1990) and most of ingested PQ is eliminated within 24 h (Hawksworth et al., 1981).

The exact mechanisms involved in PQ-induced renal dysfunction and associated acute renal failure are not yet fully established, but increasing evidences suggest that reactive oxygen species (ROS) play a role (Samai et al., 2007; Dinis-Oliveira et al., 2009; Park et al., 2010). Although the generation of ROS can trigger an apoptotic response, a direct association between PQ-induced nephrotoxicity and apoptosis has not been completely established. The general pathway of apoptosis is activated by apoptosis genes and the caspase cascade. The activation of caspase-3 plays critical roles initiating and executing apoptosis (Earnshaw et al., 1999). PQ-induced apoptosis has been mainly investigated in association with caspase-3 in dopaminergic neurons (Peng et al., 2004; Hou et al., 2008), cerebellar granule cells (Gonzalez-Polo et al., 2004), and the lungs (Dinis-Oliveira et al., 2007).

Based on the role of ROS, antioxidants may be an important tool against PQ-induced toxicity due to the lack of effective treatments or a specific antidote (Daisley and Hutchinson, 1998). The effects of PQ on antioxidant system and the role of antioxidants in PQ

* Corresponding authors. Address: Department of Anatomy, School of Medicine, Jeju National University, Jeju-Do 690-756, Republic of Korea. Tel.: +82 64 754 3823; fax: +82 64 725 2593 (S.P. Yoon), tel.: +82 62 370 7889; fax: +82 62 371 3092 (B.C. Shin).
E-mail addresses: spyoon@jejunu.ac.kr (S.P. Yoon), bcshin@chosun.ac.kr (B.C. Shin).

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toxicity have been evaluated (Ray et al., 2007). Positive effects on PQ toxicity have been reported with superoxide dismutase mimetics (Samai et al., 2007), lysine acetylsalicylate (Dinis-Oliveira et al., 2009), and quercetin (Park et al., 2010). Chitin, chitosan, and chitosan oligosaccharide (COS) have received interest as useful material of natural resources (Singla and Chawla, 2001; Shahidi and Abuzaytoun, 2005). These compounds have a wide variety of physiological activities such as wound healing, anti-hypercholesterolemic, anti-tumor, anti-ulcer, anti-microbial effects, and anti-oxidant effects. COS has water-soluble and absorbable properties compared with chitin and chitosan and shows free radical scavenging activity *in vitro* (Je and Kim, 2006; Wu and Tsai, 2007). Recent reports on COS have shown positive anti-oxidant effects for glycerol-induced acute renal failure (Yoon et al., 2008), carbon tetrachloride-induced liver damage (Yan et al., 2006), and diabetic pancreatic islets (Yuan et al., 2009; Kim et al., 2009), indicating that COS may be useful as a treatment strategy for PQ toxicity.

Therefore, we investigated whether PQ-induced nephrotoxicity is involved in apoptosis and whether COS protects rats from PQ-induced nephrotoxicity. First, the apoptotic gene p53 upregulated modulator of apoptosis (PUMA) and an effector of the caspase cascade, caspase-3, were evaluated to gain insight into apoptotic signaling during PQ-induced nephrotoxicity. Second, the protective effects of COS were estimated through restoration of serological parameters and by PUMA and caspase-3 expression. Finally, the roles of apurinic/apyrimidinic endonuclease (APE) were also assessed in PQ-induced nephrotoxicity. APE is a regulator of apoptosis and is associated with base excision repair (Bernstein et al., 2002; Tell et al., 2009). The results of this study will aid in further understanding of PQ-induced apoptosis in the rat kidney and may provide a new therapeutic strategy.

2. Materials and methods

2.1. Chemicals

PQ was purchased from Sigma–Aldrich (St. Louis, MO, USA). Low molecular weight chitosan (>98% deacetylated, <10 cps viscosity) was purchased from YB bio (Gyungbuk, Republic of Korea) and COS was obtained from the low molecular weight chitosan by the enzymatic method (Muraki et al., 1993; Kim et al., 2009). All other chemicals were the highest grades of commercially available materials.

2.2. Animals and treatments

PQ toxicity model (Willis et al., 2007) and dose of COS (Kim et al., 2009) were followed as previously described. Male Sprague–Dawley rats (Da-mool Science, Daejeon, Republic of Korea; 8–10 weeks old, 200–250 g) were daily pretreated with normal saline or COS (500 mg/kg, p.o.) 3 days before PQ administration. All animals were injected intraperitoneally with PQ (60 mg/kg). They were divided into control group and COS group and sacrificed before PQ administration (n = 5/each group) and 4, 12, and 24 h after PQ injection (n = 7/each group). All experimental procedures and care of animals were conducted in accordance with the guidelines of Jeju National University's Animal Care and Use Committee.

2.3. Biochemical analysis

Changes on body weight were checked through experimental period and 24 h urine was collected. Blood samples were collected via the right ventricle to determine PQ concentrations, serum blood urea nitrogen (BUN), and creatinine. The PQ concentrations were measured with a colorimetric method with an alkaline sodium dithionite solution (Green Cross Reference Laboratories, Gyunggi-Do, Korea) in whole blood. BUN and creatinine levels were measured using a biochemical autoanalyzer (AVIDA 1650™, Bayer, Tarrytown, NY, USA).

2.4. Antibodies

The primary antibodies used in this study were monoclonal anti-APE (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-caspase-3 (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), polyclonal anti-cleaved caspase-3 (1:1000; Cell Signaling Technology, Inc.), polyclonal anti-PUMA (1:1000; Santa Cruz Biotechnology, Inc.), and polyclonal anti-β-actin (1:1000; Santa Cruz Biotechnology, Inc.).

2.5. Western blot assay

Isolated kidney sections were suspended in 1 mL of cold homogenizing buffer [20 mM Hepes (pH 7.4), 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 g/mL leupeptin, 10 g/mL aprotinin, 1 mM Na_3VO_4, and 5 mM NaF] containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and were homogenized using a ultrasonic cell disruptor (Branson Ultrasonics Co., Danbury, CT) for 30 s three times, with a 30 s interval, and centrifuged at 10,000g for 10 min at 4 °C. Protein concentration in the supernatants was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An aliquot of the supernatant (30 μg protein) was then suspended in 20 μl of a loading buffer comprised of a 1:1 mixture (v/v) of the above homogenizing buffer and a sample buffer [50 mM Tris–HCl (pH 6.5), 0.5 mg/mL bromophenol blue, 10% glycerol, 10% SDS, and 1% β-mercaptoethanol]. It was then boiled for 5 min at 100 °C, electrophoresed on 10% SDS–PAGE gels, and transferred to polyvinyldifluoridine membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Immunoblotting was carried out with each primary antibody. Appropriate secondary antibodies linked to horseradish peroxidase (GE Healthcare Bio-Sciences Corp.) were diluted at 1:4000. The blotted proteins were then detected using the iNtRON Biotech Enhanced Chemiluminescence Detect System (Seoul, Republic of Korea) and quantified using ImageQuant 350 (GE Healthcare Korea, Seoul, Republic of Korea). The data expressed as densitometric units of each primary antibody relative to β-actin and in reference to the value of the control sample for each gel.

2.6. Cytoarchitecture and Immunohistochemistry

After fixation with 4% paraformaldehyde, the kidneys were embedded in paraffin wax (Tissue-Tek, Sakura, Japan) using standard procedures. Next, 5-μm-thick serial sections were cut using a Leica RM 2155 rotary microtome (Nussloch, Germany) and mounted on slides coated with 3-aminopropyl-tri-ethoxy-silane (Sigma–Aldrich, St. Louis, MO). Randomly selected samples were stained with periodic acid-Schiff using routine protocol.

Immunohistochemical staining was carried out by the routine method. In brief, incubation with primary antibodies was performed for 48 h at 4 °C. The binding was visualized using an ImmPRESS™ avidin–biotin-peroxidase kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Omission of incubation with the primary or secondary antibody served as a control for false-positives. Immunolabelled images were captured directly using a C-4040Z digital camera and Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan). The captured images were saved and subsequently processed using Adobe Photoshop (Adobe System, San Jose, CA). The brightness and contrast of the images were adjusted only for the purpose of background consistency.

2.7. Statistical analysis

Results were expressed as mean ± SD. The statistical differences between groups were analyzed using Mann–Whitney test and Kruskal–Wallis test (SPSS version 11.0, Chicago, IL, USA). A p value of less than 0.05 was considered significant.

3. Results

3.1. Body weight and serological aspects (Table 1)

Body weight decreased in both control and COS groups in a time-dependant manner after the PQ injection. The COS group significantly lost body weight 24 h (21.3 ± 2.31 g) after the PQ injection when compared to the control group (15.0 ± 3.46 g).

PQ concentration increased in the control group and decreased considerably in the COS group in a time-dependant manner. Serum PQ concentration in the COS group was higher at 4 h after PQ injection than that in control group (4.30 ± 1.07 μg/mL vs 2.67 ± 0.27 μg/mL), but significantly lower at 12 h (0.93 ± 0.08 μg/mL) and 24 h (0.09 ± 0.09 μg/mL) after PQ injection compared to control group (3.44 ± 2.36 μg/mL and 3.39 ± 3.18 μg/mL, respectively).

BUN and serum creatinine levels increased in the control group in a time-dependant manner, but did not change considerably in COS group. BUN and creatinine in the COS group were significantly lower at 12 h (24.3 ± 4.9 and 0.50 ± 0.00 mg/dL) and 24 h (21.8 ± 2.8 and 0.40 ± 0.00 mg/dL) after PQ injection compared to control group (50.4 ± 20.5 mg/dL and 0.96 ± 0.29 mg/dL, 89.4 ± 20.3 mg/dL and 1.20 ± 0.40 mg/dL, respectively).

The 24 h urine volume tended to decrease slightly in the control group and increase in the COS group after PQ injection, but the