

Comparative Evaluation of Garlic (S-allylcysteine) and Reduced Glutathione in Mitigating the Toxopathologic Effects of Cyclophosphamide in Urinary Bladder

Kanchan Bhatia*, Diana Ali Hmoud Al-Quwaie,
Nizamuddin Farooqui and Saif Ahmad

Department of Biological Sciences, Rabigh College of Science and Arts,
King Abdulaziz University, Rabigh, Saudi Arabia.

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Garlic supplements have shown promise in the treatment of various diseases including the urinary bladder toxicity by cyclophosphamide (CP). Aged garlic extract, which contains S-allylcysteine as the bioactive sulfur compound, in particular is standardizable and highly tolerable, with little or no known harmful interactions. Here we describe biologically plausible mechanisms of garlic's lowering of CP toxicity in urinary bladder compared to purified reduced glutathione that is a cellular antioxidant. Thiols are recognised as key components involved in the maintenance of redox balance. Their antioxidant activity may be both enzymatic and non-enzymatic. Both S-allylcysteine (SAC) as well as reduced glutathione (GSH) are sulphur containing compounds with antioxidant activity. Administration of SAC and GSH (150 mg/kg/bw) administration in CP treated animals demonstrated comparable lowering of lipid peroxidation as well as increased GSH levels in the urinary bladder. Since oxidative stress mediated apoptosis is associated with CP urotoxicity, these toxic manifestations can be alleviated by use of SAC as we have already shown its mitigating effect on oxidative stress. Immunostaining of urinary bladder sections with Bax, Bcl2, Cyt c showed the protection mediated as result of administration of SAC against CP-mediated apoptosis. Thus, the study shows that administration of SAC mitigated CP- induced urotoxicity by controlling oxidative stress and apoptosis in urothelium.

Key words: Reduced Glutathione, S-allylcysteine, oxidative stress, apoptosis, protection.

Garlic (*Allium sativum*) has been utilized for the maintenance of health and treatment of disease for many years. Among the different types of garlic preparations that are available, many thiol containing components have been described including S-allylcysteine (SAC)^{1,2}. Of the major products of garlic, SAC, the main active compound in aged garlic extract, is stable and standardizable, and has been found to be highly tolerable^{3,4}. Reports on the antioxidant properties of SAC have been published in various pathologies and toxicity

studies^{5,6,7}. Our previous publication has shown protective effect of SAC on cyclophosphamide (CP) induced hemorrhagic cystitis, a side effect of use of CP as an antineoplastic drug⁸. This protection mediated by SAC is due to the restored glutathione levels and dependent as CP causes urinary bladder toxicity via depletion of GSH and its dependent enzymes via its active metabolite, acrolein and free radicals results in the hemorrhage, edema and tissue necrosis^{9,10,11}. Both ROS and RNS play an important role in cell pathophysiological functions as well as cell signaling. As a result of the overproduction of reactive oxygen species ROS and RNS during inflammation leads to an extensive oxidative stress and cellular injury via both

* To whom all correspondence should be addressed.
Tel.: +966583686988;
E-mail: kanchan_bhatia2002@yahoo.com

apoptosis through several mechanisms^{12,13,14}. Apoptosis also plays a significant role in CP urotoxicity. As shown by Jezernik *et al.*¹⁵ that during 24h after administration of CP, apoptosis and increased iNOS activity contribute significantly to the damage to the uroepithelium even in the absence of necrotic cells. This is followed by rapid regeneration of the epithelium lining after 24h. Thus, the role of antioxidants seems inevitable in the prevention of this free radical assault^{8,16}. In the present study, we did comparative evaluation of both SAC and reduced glutathione as mitigators of oxidative stress role of SAC in prevention of CP-induced apoptosis in urinary bladder.

MATERIALS AND METHODS

Chemicals

Antibodies for Bax, Bcl2 and Cytc were obtained from Santa Cruz Biotech (USA). S-allylcysteine ($C_6H_{11}NO_2S$) in powder form was gifted by Sami Labs, India.

Animals

The study was conducted in Swiss albino male mice (25 ± 2 g) and was approved by the Institutional Animal Ethics Committee. The animals were bred and maintained under the standard laboratory conditions (temperature 25 ± 2 °C; photoperiod of 12 h). Commercial pellet diet and water were given *ad libitum*.

Dose treatment schedule

Animals were divided in six groups CP, GSH and SAC were suspended in normal saline. Animals were divided in six groups ($n = 6$ each group). The dose and treatment schedule are given in Table 1. Dosing was done in such a manner that all the animals could be sacrificed on the same day.

Urinary bladders were removed after animals were sacrificed after 24 h by cervical dislocation under mild anesthesia and used for the measurement of different parameters.

Biochemical investigations

After termination of treatment, animals were sacrificed under mild anesthesia and their bladders were removed. The bladder tissue was homogenized to obtain the post-mitochondrial supernatant (PMS).

LPO

LPO was measured using the procedure of Uchiyama and Mihara (1978)^{8,17} and the data

was expressed as % change in LPO levels.

GSH

GSH was measured by the method of Haque *et al.* (2003)^{8,17} and expressed as % change in GSH levels.

Statistical analysis

Data are expressed as means \pm SE. Single factor one-way analysis of variance (ANOVA) was applied to determine significant difference in observation of CP group with control. Student-Newman-Keuls test was applied for analyzing the significance between different treatment groups. *P* values < 0.05 were considered significant.

Immunohistological Investigation

Separate set of animals were treated with same doses as given in Table 1 and sacrificed at 18 h of CP administration for estimation¹⁶. The bladder was fixed in 10% buffered-formalin. After fixation was completed (minimum time- 18-24 h), the tissues were trimmed into 2-4 mm thick sections for processing and sectioning. Paraffin embedded cross-sections of 4-5nm thickness were mounted on slides coated with 10% L-polylysine. Post deparaffinisation, antigen retrieval was done with citrate buffer (0.05M, pH6.0) for 30 min at 100° C. Blocking was done for 10 min followed by incubation with primary antibody at 1:50 dilution (Bax, Bcl2, Cytc) for 1 h at room temp. Washed with Tris buffer 3 times and then added the secondary antibody for 30 min. The slides were then washed 3 times with Tris buffer and stained with DAB (Dako) for 2 min. Then sections were counterstained with Hematoxylin mounted with (DPX) mountant. The slides were observed for histological changes and microphotographs were taken using Olympus BX50 microscope¹⁸.

RESULTS

Mortality and other toxicity signs and symptoms

No treatment related mortality was observed in any of the groups of animals and there were no toxicity signs and symptoms, which could be attributed to treatments.

Comparative effect of SAC and GSH on lipid peroxidation and reduced glutathione

CP treatment increased LPO levels by 28% in bladder over control values (Fig. 1). Both SAC and GSH treatment in CP-treated animals showed reduction in LPO in bladder. No significant

Table 1. Different dose groups and dose schedule

Experimental group	Treatment and dose schedule
Control	Normal saline i.p. for 2 days
CP	CP (200 mg/kg bw, i.p.)
SAC + GSH	SAC (150 mg/kg bw, i.p. twice) at 12 h interval GSH (150 mg/kg bw, i.p. twice) at 12 h interval
SAC + CP	SAC (150 mg/kg bw, i.p., twice at 12 h interval) along with CP (200 mg/kg bw, i.p., single dose) 1 h after SAC administration
GSH + CP	GSH (150 mg/kg bw, i.p., twice at 12 h interval) along with CP (200 mg/kg bw, i.p., single dose) 1 h after GSH administration

Table 2. Efficacy of SAC on CP-induced apoptosis in Urinary epithelium

Group	Bax	Bcl2	Cytc
Control	-	+	-
CP	+++	++	+++
SAC	-	+	++
SAC+CP	+	+	++

Score interpretation: - no stain, + mild, ++ moderate, +++ high

change in LPO induction in SAC+CP when compared to GSH+CP group (31% vs 26%). Cellular GSH content decreased in urinary bladder significantly ($P < 0.001$) in CP-treated animals as compared to controls (Fig. 2). Both SAC and GSH treatment along with CP increased level of GSH as compared to CP treated animals (60% vs 56%). No significant change was observed in GSH content when SAC+CP group was compared with GSH+CP group.

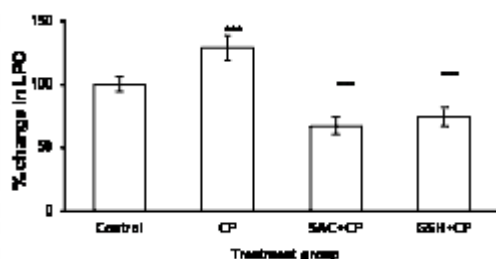


Fig.1. Comparative efficacy of SAC and GSH on LPO in urinary bladder. Significant differences are indicated by *** $P < 0.001$ in CP treated animals when compared with control animals and *** $P < 0.001$ and *** $P < 0.001$ indicates significant changes in SAC+CP and GSH+CP group when compared to CP group. Values are expressed as means \pm S.E. ($n=6$).

Immunohistological Investigations

Fig. 3(a-c) shows 18h of CP treatment resulted in increased levels of Bax, Bcl2 and Cytc in the uroepithelial cells when compared to control sections (Fig. 3d-f). However, on administration of SAC in CP-treated animals decreased expression of Bax and Cytc was observed along with Bcl2 when compared to CP- treated animals (Fig. 3g-i). In SAC group, no positive staining was observed in Bax but moderate staining in Bcl2 and Cyt c was seen (data not shown) Table 3.

DISCUSSION

CP mediated urinary bladder toxicity is dose dependent and results in decrease in thiol status and increased oxidative stress. Increased levels of protein carbonyls have also been reported. As seen in our results also that high dose (200 mg/kgbw) of CP resulted in increased levels of TBARS

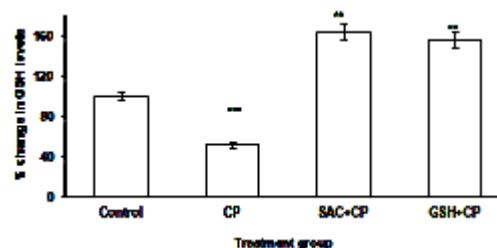


Fig. 2. Comparative efficacy of SAC and GSH on CAT activity in urinary bladder. Significant differences are indicated by *** $P < 0.001$ in CP treated animals when compared with control animals and ** $P < 0.01$ indicates significant changes in SAC+CP when compared to CP group. Values are expressed as means \pm S.E. ($n=6$).

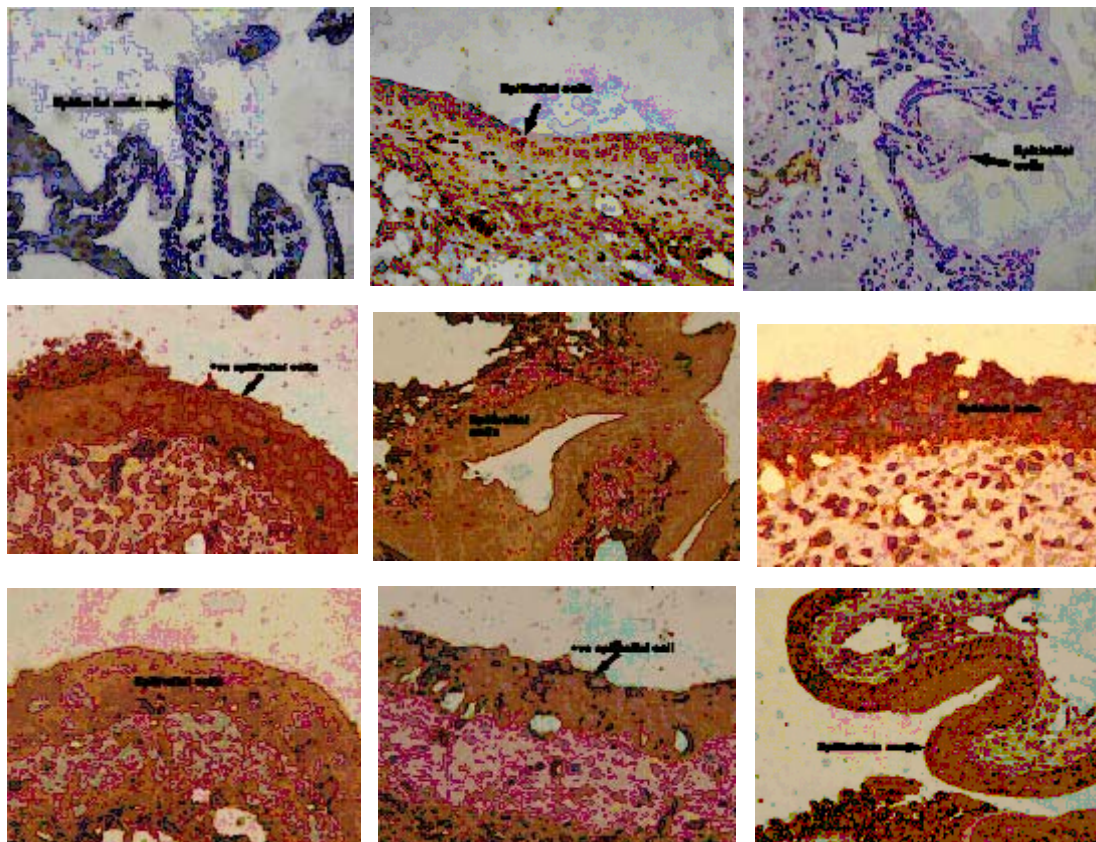


Fig.3. Immunohistochemistry of apoptosis markers (Bax, Bcl2 and Cyt c, respectively) in urinary bladder of mice from different treatment groups (100X). Sections (A-C) shows CP treatment resulted in increased levels of Bax and Cyt c in the uroepithelial cells when compared to control sections (D-F). SAC+CP-treated sections have lower expression of Bax , Cyt c when compared to CP- treated animals (G-I).

and reduced levels of GSH significantly. As reported in our previous publication, we found that SAC mitigated oxidative stress by lowering LPO and increasing reduced glutathione levels⁸. In addition, administration of exogenous glutathione has also been shown to prevent CP urotoxicity^{10,16,19}. From above results when we compared the efficacy of SAC vs. reduced glutathione, the degree of protection offered by these drugs was same. Both SAC and reduced glutathione compensate the decreased GSH levels in urinary bladder of CP-treated mice to comparable levels. This suggests that SAC is in par with glutathione as a potent antioxidant. Since we have already published that SAC mediated lowering of CP induced oxidative stress also improved the histological manifestations of CP, we further investigated the role of apoptosis in CP urotoxicity

and if SAC can mitigate this effect.

CP mediated apoptosis is a major contributor to urinary bladder toxicity inflicted by CP. Exposure to CP has been shown to cause destruction of the urothelium with increased rate of apoptosis at 18 h in acute phase studies^{16,20}. Immunostaining of well established biomarkers of apoptosis i.e Bax and Cyt c were remarkably increased in the uroepithelium sections of CP treated mice when compared to controls as reported previously^{21,22,23}. However, Bcl2 also showed increased levels on CP-treatment suggesting possibility of compensatory mechanism or due to rapid regeneration of epithelium. Similar trend has been reported by Juaristi *et al.*²⁴ in CP-treated bone marrow cells. Administration of SAC in CP-treated mice resulted in decreased levels of Bax, Cyt c and also Bcl2 expression. The decreased levels of Bcl2

may be due to less regeneration as a result of protective action of SAC in CP-treated animals. SAC group showed lower levels of Bcl2 and Cyt c and no Bax expression. These changes show the involvement of mitochondrial mediated induction of apoptosis as mitochondria are the site of peroxynitrite formation due to enhanced superoxide and presence of nitric oxide in pathological conditions^{25,26}. However, a caspase dependent pathway should also be considered. These findings demonstrate a strong potential of SAC in modulating CP-induced urotoxicity by regulating apoptosis and oxidative stress. The present findings suggest a further detailed investigation into these compounds to establish its clinical efficacy.

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