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Research article



# S-allylmercaptocysteine scavenges hydroxyl radical and singlet oxygen *in vitro* and attenuates gentamicin-induced oxidative and nitrosative stress and renal damage *in vivo*

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## Abstract

**Background:** Oxidative and nitrosative stress have been involved in gentamicin-induced nephrotoxicity. The purpose of this work was to study the effect of S-allylmercaptocysteine, a garlic derived compound, on gentamicin-induced oxidative and nitrosative stress and nephrotoxicity. In addition, the *in vitro* reactive oxygen species scavenging properties of S-allylmercaptocysteine were studied.

**Results:** S-allylmercaptocysteine was able to scavenge hydroxyl radicals and singlet oxygen *in vitro*. In rats treated with gentamicin (70 mg/Kg body weight, subcutaneously, every 12 h, for 4 days), renal oxidative stress was made evident by the increase in protein carbonyl content and 4-hydroxy-2-nonenal, and the nitrosative stress was made evident by the increase in 3-nitrotyrosine. In addition, gentamicin-induced nephrotoxicity was evident by the: (1) decrease in creatinine clearance and in activity of circulating glutathione peroxidase, and (2) increase in urinary excretion of N-acetyl-β-D-glucosaminidase, and (3) necrosis of proximal tubular cells. Gentamicin-induced oxidative and nitrosative stress and nephrotoxicity were attenuated by S-allylmercaptocysteine treatment (100 mg/Kg body weight, intragastrically, 24 h before the first dose of gentamicin and 50 mg/Kg body weight, intragastrically, every 12 h, for 4 days along gentamicin-treatment).

**Conclusion:** In conclusion, S-allylmercaptocysteine is able to scavenge hydroxyl radicals and singlet oxygen *in vitro* and to ameliorate the gentamicin-induced nephrotoxicity and oxidative and nitrosative stress *in vivo*.

## Background

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in several renal diseases [1-8] including the renal damage induced by the antibiotic gentamicin (GM) [9-19]. GM induces superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (HO) production from renal mitochondria [13,14]. In addition, $H_2O_2$ generation [15], lipoperoxidation [16,17], and the content of nitrotyrosine [10-12], and protein carbonyl [10,18] are increased and that of reduced glutathione is diminished [18] in renal cortex from GM-treated rats. Moreover, the administration of several compounds with antioxidant properties, ROS scavengers, and/or antioxidant enzymes are able to ameliorate the severity of GM-induced renal damage [9,10,16-19]. In addition, the kidneys from GM-treated rats are more vulnerable to ROS because of they are deficient in the antioxidant enzymes Mn-superoxide dismutase (Mn-SOD) [10,17], glutathione peroxidase (GPx) [17], glutathione reductase (GR) [10], and catalase (CAT) [17].

On the other hand, S-allylmercaptocysteine (SAMC) ($CH_2$=CH-$CH_2$-S-S-$CH_2$-CH-$NH_2$-COOH) is one of the water soluble organosulfur compounds found in aged garlic extract (AGE) which is obtained by ethanol extraction of sliced garlic bulbs [20]. It has been postulated that SAMC may be one of the active compounds responsible for the protective effect of AGE observed in several experimental models associated to oxidative stress [10,21-27]. It has been clearly shown that SAMC has *in vitro* [21,22] and *in vivo* [23] antioxidant properties. SAMC is able to (a) inhibit lipoperoxidation [22,23] and low density lipoprotein oxidation [21] and (b) scavenge 1,1-diphenyl-2-picrylhydrazyl radical [22]. Moreover, SAMC pretreatment protects liver against the damage induced by acetaminophen [23-25], carbon tetrachloride [25,26], and D-galactosamine [26].

Based on the above mentioned data, the hypothesis was made that SAMC could ameliorate GM-induced oxidative and nitrosative stress and renal damage. In this paper the ROS scavenging properties of SAMC, and the effect of this compound on GM-induced (a) renal damage, (b) oxidative and nitrosative stress, and (c) on activity of antioxidant enzymes (Mn-SOD, GPx, GR, and CAT) in rats are presented. Renal damage was evaluated by measuring glomerular and tubular function and by histological analysis [3,6,7,10]. Nitrosative stress was evaluated by measuring nitrated proteins by immunohistochemistry using antibodies against 3-nitrotyrosine (3-NT) [1,2,7,10-12]. Oxidative stress was evaluated by measuring protein carbonyl content (by immunohistochemical [27] and spectrophotometric methods [10,28]) and 4-hydroxy-2-nonenal (4-HNE) protein adducts (by immunohistochemistry [29,30]). Protein oxidation by immunohistochemistry was performed using antibodies against dinitrophenol (DNP) [27].

## Results

### In vitro *ROS scavenging properties of SAMC*

SAMC was able to scavenge hydroxyl radicals and singlet oxygen ($^1O_2$) in a dose-dependent way, these effects become significant at 0.3 mM and 1.25 mM, respectively (Figs. 1A and 1B). In contrast, SAMC was unable to scavenge superoxide anion and hydrogen peroxide: Fig. 1 [see Additional file 1].

### *In vivo studies*

#### *Body weight, food intake, and urinary volume*

Body weight and food intake were not statistically different among the four groups of rats at the end of study (ANOVA, p = 0.3194 and 0.3842, respectively, Table 1). Urinary volume increased significantly in the GM group, and SAMC was unable to prevent this increase in the GM+SAMC group (Table 1).

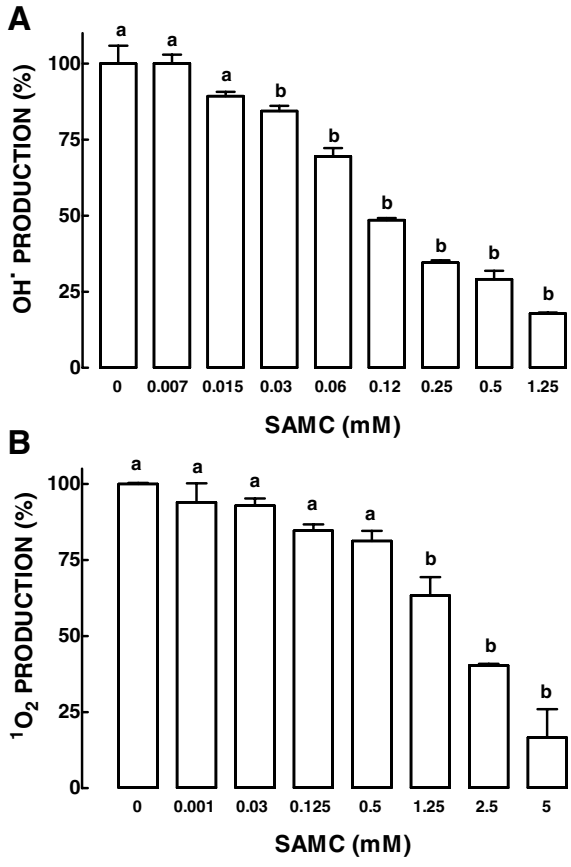


**Figure 1**
Ability of SAMC to scavenge hydroxyl radical (A) and to quench singlet oxygen (B). Data are mean ± SEM. Bars with different letter are significantly different (P < 0.001). *n* = 3–4.

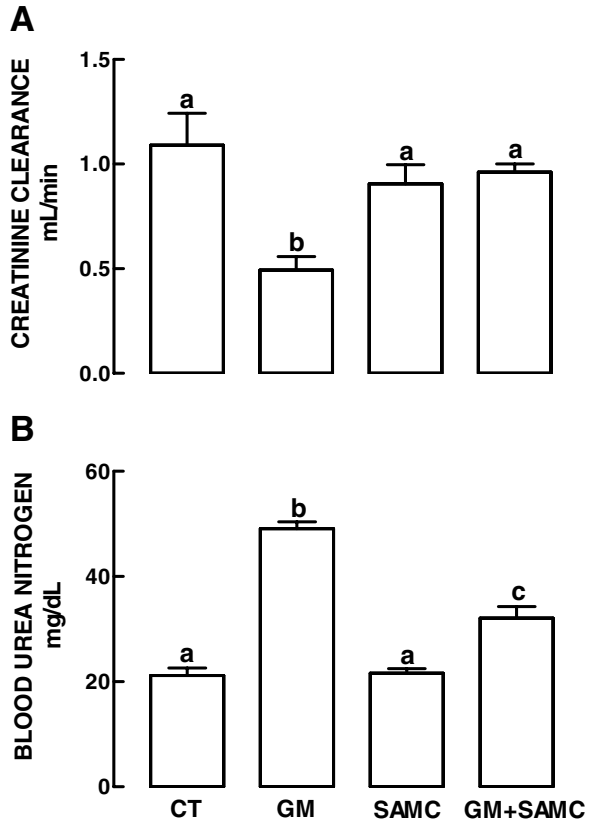


**Figure 2**
Creatinine clearance (A) and blood urea nitrogen (B) on day 5 in the four groups of rats studied: CT: control group, GM: gentamicin group, SAMC: S-allylmercaptocysteine group, and GM+SAMC: gentamicin+S-allylmercaptocysteine group. Data are mean ± SEM. Groups with different letter are significantly different ($P < 0.01$). $n$ = 4–5.

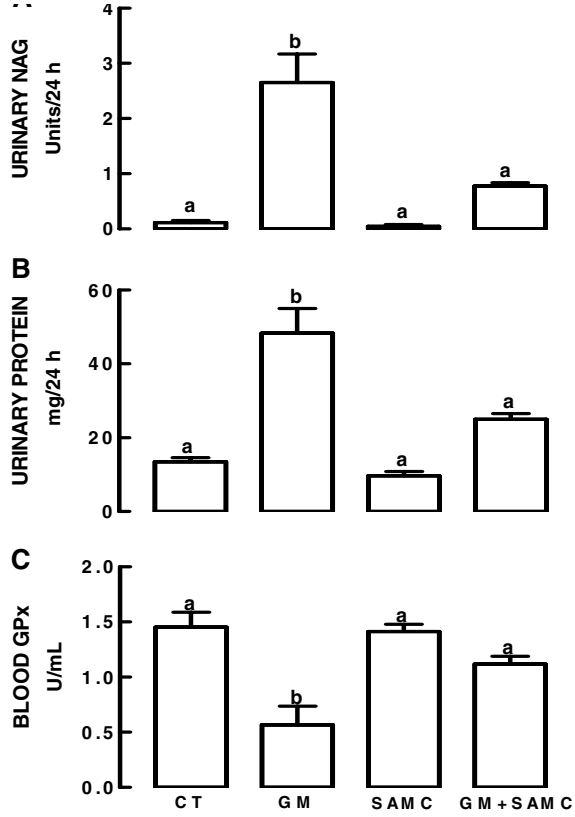


**Figure 3**
Urinary excretion of NAG (A) and total protein (B), and blood GPx activity (C) in the four groups of rats studied. Data are mean ± SEM. Groups with different letter are significantly different ($P < 0.001$). $n$ = 4–5.

*Markers of glomerular and tubular damage*

Creatinine clearance decreased 55% and blood urea nitrogen (BUN) increased 2.3 fold in the GM group compared to the control (CT) one (Fig. 2A and 2B). SAMC prevented the decrease in creatinine clearance and attenuated the increase in BUN in the GM+SAMC group. GM increased significantly urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) and total protein (Figs. 3A and 3B). The increase in both parameters was prevented by SAMC. Blood GPx activity diminished 61% in the GM group and the treatment with SAMC was able to prevent this reduction in the GM+SAMC group (Fig. 3C). Creatinine clearance, BUN, urinary excretion of both NAG and total protein, and blood GPx activity were similar in the CT and SAMC groups (Figs. 2 and 3).

*Histological analysis*

Rats treated with GM showed vacuolization and necrosis in the proximal tubular epithelial cells (Fig. 4). The percentage of damaged tubular area in the GM group was of 77 ± 7% and the treatment with SAMC significantly decreased this percentage in the GM+SAMC group to 38 ± 5% ($p = 0.021$). There were not renal histological alterations in CT and SAMC groups (Fig. 4). Therefore, SAMC treatment attenuated not only functional impairment but also structural alterations induced by GM.

*3-nitrotyrosine (3-NT), dinitrophenol (DNP), and 4-hydroxy-2-nonenal (4-HNE) immunostaining*

Representative images showing an increase in 3-NT, DNP, and 4-HNE immunostaining in GM-treated rats are showed in Figs. 5, 6, and 7, respectively. These increases

**Table 1: Body weight, food intake, and urinary volume in the 4 groups of rats studied on day 5**

| | Control | Gentamicin | SAMC | Gentamicin+SAMC |
|---|---|---|---|---|
| Body weight (g) | 259 ± 2.1 | 252 ± 4.3 | 263 ± 4.7 | 255 ± 4.3 |
| Food intake (g/24 h) | 21.2 ± 2.3 | 18.0 ± 1.8 | 21.8 ± 0.4 | 18.6 ± 2.2 |
| Urinary volume(ml/24 h) | 6.5 ± 1.1 | 18.6 ± 2.6[a] | 7.1 ± 1.0 | 22.1 ± 2.4[a] |

Values are mean ± SEM; SAMC, S-allylmercaptocysteine. [a]$p < 0.01$ *vs.* CT, n = 4–5

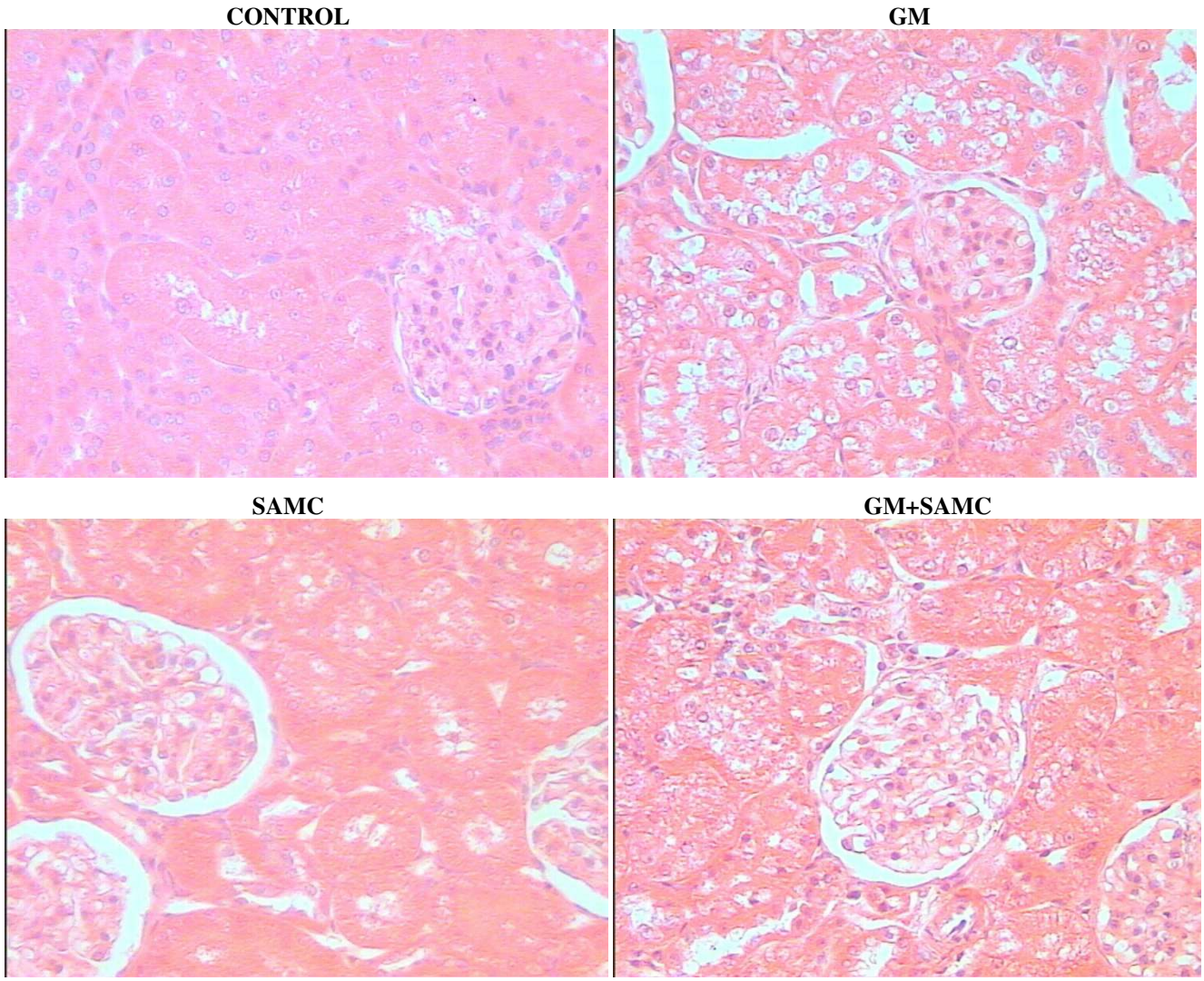


**Figure 4**
Representative light microscopic findings in the renal cortex from the four groups of rats studied on day 5. GM-treated rats showed necrosis and vacuolization in the proximal tubular epithelial cells. Histological damage decreased in the GM+SAMC group. H&E. 200×.

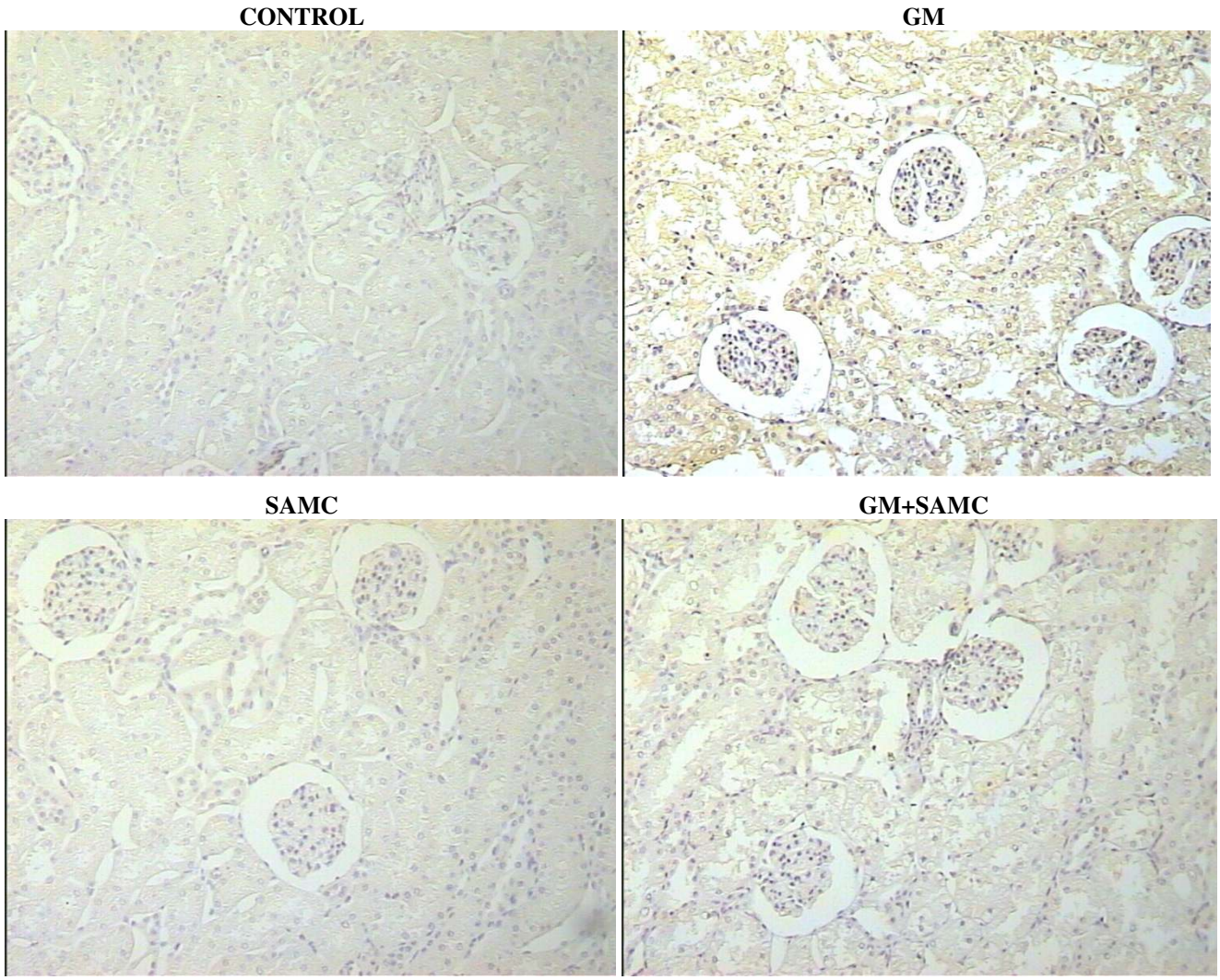


**Figure 5**
Immunohistochemistry for 3-nitrotyrosine (3-NT) in renal cortex. 3-NT immunostaining is increased in GM group and this increase is partially prevented in GM+SAMC group.

were attenuated by SAMC pretreatment in the GM+SAMC group (Figs. 5, 6, 7). Only proximal tubules were positive for 3-NT, DNP, and 4-HNE.

*Spectrophotometric assay of protein carbonyl content*

GM treatment induced a 1.5-fold increase in the protein carbonyl content in renal cortex compared to the CT group (Fig. 8). The increase induced by GM was prevented by SAMC (GM+SAMC group). The protein carbonyl content was similar in CT and SAMC groups.

*Antioxidant enzymes in renal cortex*

The activities of Mn-superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT) decreased 38%, 44%, 10%, and 47%, respectively in GM-treated rats (Table 2). The decrease in Mn-SOD did not reach statistical significance. Mn-SOD activity was similar in CT and GM+SAMC groups. SAMC treatment attenuated but not prevented significantly the decrease in GPx activity in the GM+SAMC group. SAMC prevented the decrease in GR activity in the GM+SAMC group. CAT activity decreased 36% and 47%, respectively in SAMC and GM+SAMC groups. Cu,Zn-SOD

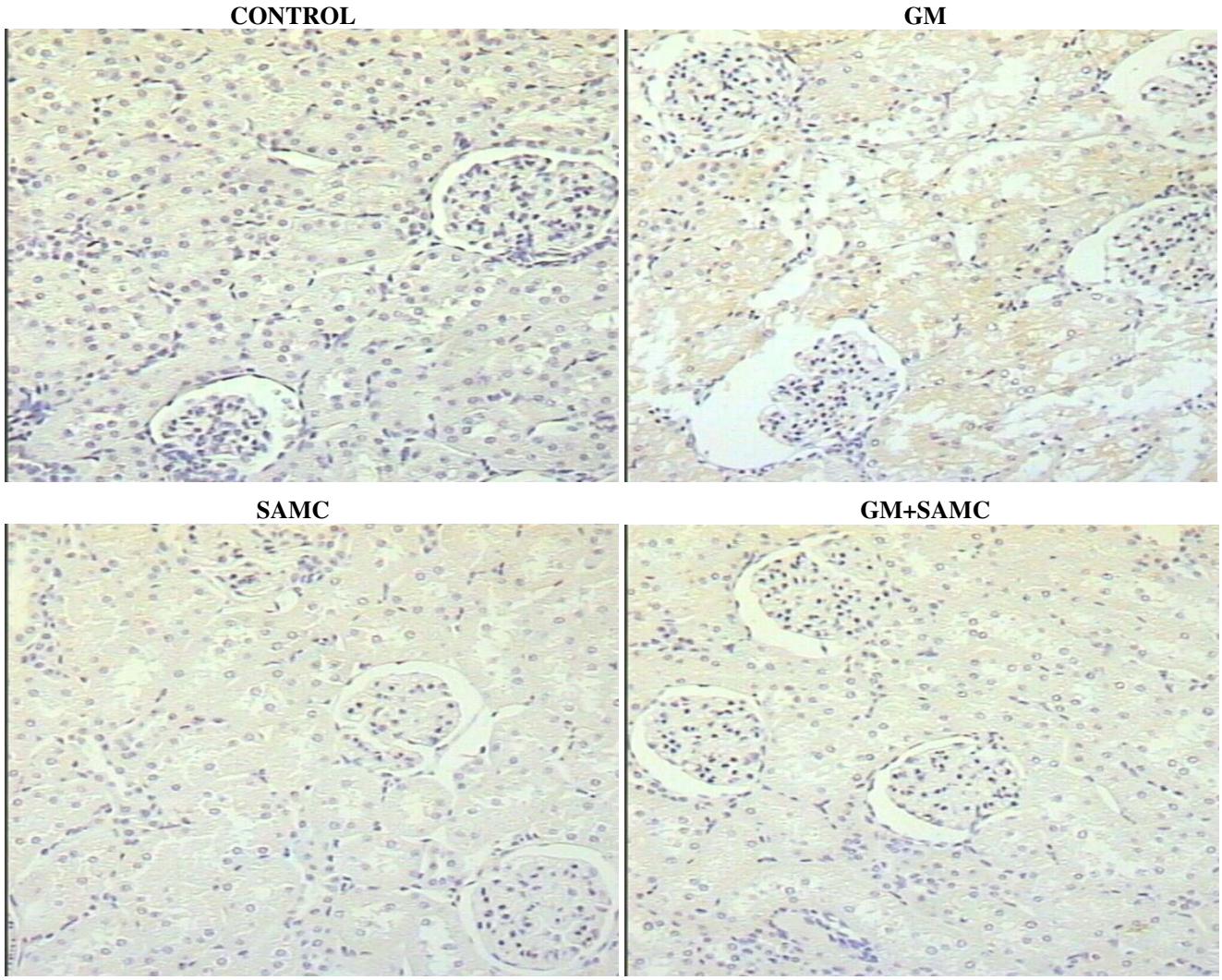


**Figure 6**
Immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE) in renal cortex. 4-HNE immunostaining is increased in GM group and this increase is partially prevented in GM+SAMC group.

remained unaffected by the GM-treatment (Table 2). The activities of all antioxidant enzymes measured in this work were not affected by SAMC (SAMC vs. CT group) (Table 2).

## Discussion

GM is an aminoglycoside widely used in clinical practice for the treatment of gram-negative infections. GM is rapidly excreted, predominantly by glomerular filtration and the reabsorption of a small but notable amount of drug by the proximal tubule results in accumulation within the renal cortex; this preferential binding is responsible for nephrotoxicity [31]. The mechanism by which GM induces nephrotoxicity remains unknown; however, it has been postulated that oxidative and nitrosative stress are involved in this process [13]. It has been shown that $O_2^-$ [11], OH [32] and $H_2O_2$ [15], are involved in renal damage induced by GM. In addition, GM induces $H_2O_2$ generation by mitochondria [14].

Interestingly, iron chelators such as deferoxamine and 2,3 dihydrobenzoic acid are able to prevent GM-induced nephrotoxicity suggesting the toxic role of iron in this disease probably by catalyzing the OH production via the

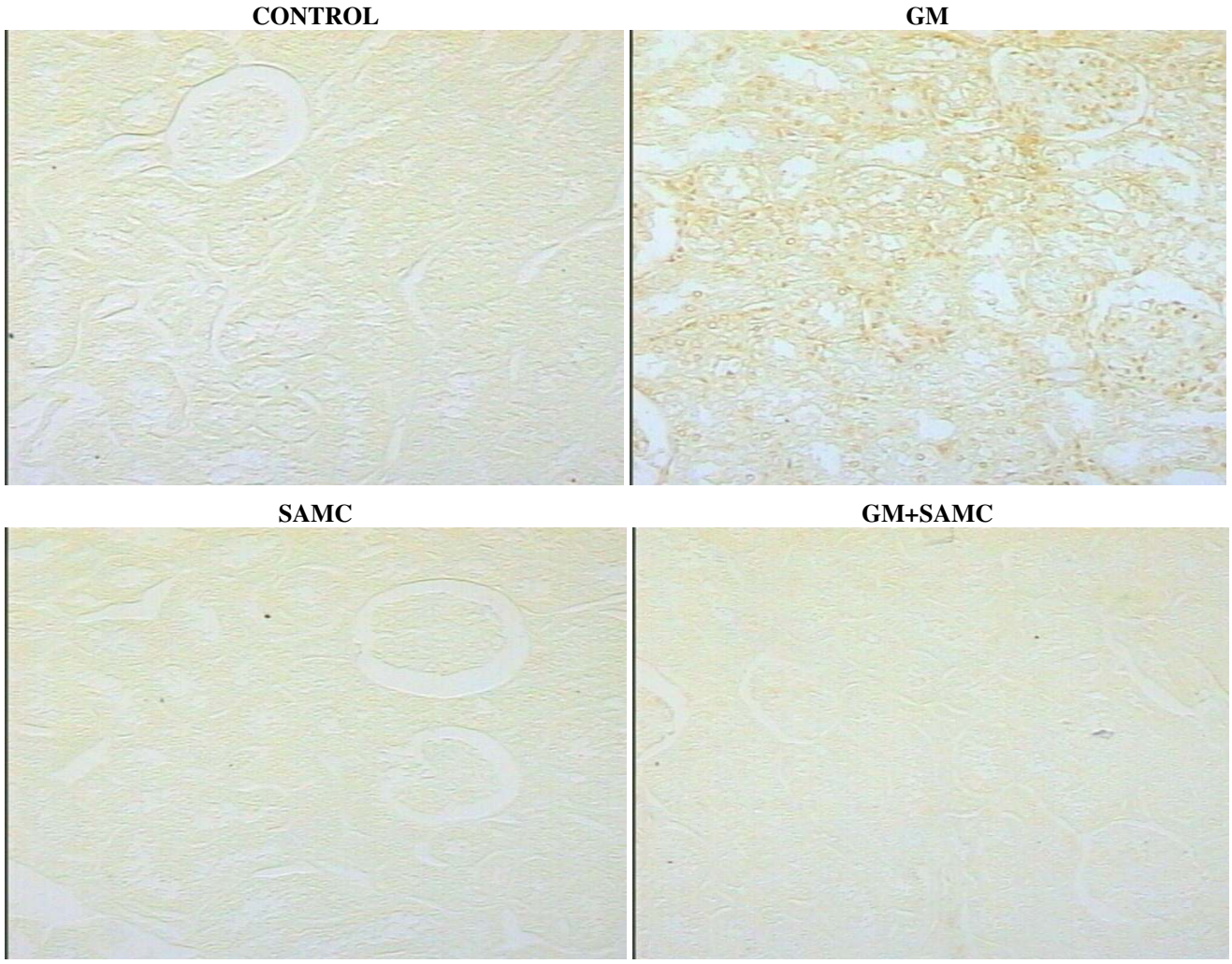


**Figure 7**
Immunohistochemistry for dinitrophenol (DNP) in renal cortex. DNP immunostaining is increased in GM group and this increase is partially prevented in GM+SAMC group.

**Table 2: Antioxidant enzymes activity in renal cortex from the four groups of rats studied on day 5**

| | Control | Gentamicin | SAMC | Gentamicin+SAMC |
|---|---|---|---|---|
| **Mn-SOD** (U/mg protein) | 7.4 ± 0.4 | 4.6 ± 0.7 | 7.8 ± 1.2 | 7.9 ± 1.4 |
| **Cu,Zn-SOD** (U/mg protein) | 26.7 ± 2.8 | 28.1 ± 2.2 | 24.1 ± 1.1 | 28.6 ± 3.4 |
| **GPx** (U/mg protein) | 0.16 ± 0.007 | 0.09 ± 0.007[a] | 0.14 ± 0.009 | 0.12 ± 0.007[a] |
| **GR** (U/mg protein) | 0.037 ± 0.002 | 0.03 ± 0.002[a] | 0.039 ± 0.002 | 0.041 ± 0.0007[b] |
| **CAT** (*k*/mg protein) | 0.55 ± 0.04 | 0.29 ± 0.03[a] | 0.35 ± 0.01[a] | 0.26 ± 0.03[a] |

Values are mean ± SEM; [a]$p < 0.05$ *vs.* CT, [b]$p < 0.001$ *vs* GM $n = 4–5$. CT, control; GM, gentamicin; SAMC, S-allylmercaptocysteine; Mn-SOD, manganese-dependent superoxide dismutase; Cu,Zn-SOD, copper/zinc-dependent superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; CAT, catalase. NOTE: The decomposition of $H_2O_2$ by CAT contained in the samples follows a first-order kinetics as given by the equation $k = 2.3/t \log Ao/A$ where $k$ is the first-order reaction rate constant, $t$ is the time over which the decrease of $H_2O_2$ due to CAT activity was measured (15 s), and $Ao$ and $A$ are the optical densities at times 0 and 15 s, respectively.

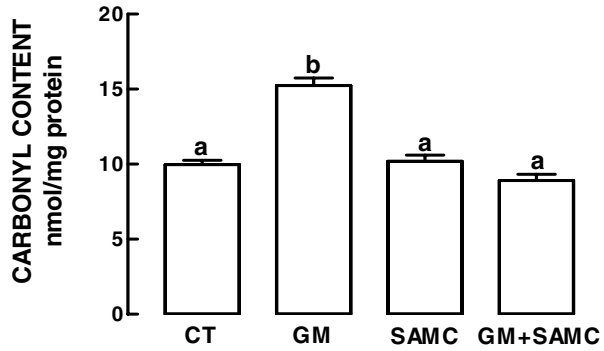


**Figure 8**
Carbonyl content in the renal cortex from the four groups of rats studied on day 5. Groups with different letter are significantly different ($P < 0.001$). $n = 4–5$.

Haber-Weiss reaction [32]. GM also induces $O_2^-$ production from mesangial cells in culture [35]. This increase was suppressed by an inhibitor of NADPH oxidase or an inhibitor of nitric oxide (NO) synthase suggesting the role of both proteins in $O_2^-$ production by GM in mesangial cells [33]. The role of OH has been evident by using OH scavengers such as dimethylthiourea, sodium benzoate and dimethyl sulfoxide [32]. The role of $O_2^-$ has been shown using exogenous administration of superoxide dismutase [34] or M40403 [11], a synthetic low molecular weight compound with SOD mimetic activity, which selectively removes $O_2^-$. In addition, it has been found that GM enhances NO generation by glomeruli and mesangial cells [35,36].

The inhibition of NO production exacerbates GM nephrotoxicity suggesting that the enhancement of NO production is very important to prevent exacerbation of renal damage in GM-induced nephrotoxicity [37,38]. On the other hand, NO is able to react with $O_2^-$ to produce peroxynitrite anion which itself is very toxic [39,40]. In addition, there is direct evidence that peroxynitrite is able to produce $^1O_2$ after reacting with linoleic acid hydroperoxide [41], suggesting a potential $^1O_2$-dependent mechanism that may contribute to cytotoxicity mediated by lipid hydroperoxides and peroxynitrite reactions in biological systems.

In addition, it has been appreciated that OH-like activities are generated from peroxynitrite [42]. The production of OH is frequently proposed to occur by the metal-catalyzed Haber-Weiss reaction. We are tempting to speculate that OH in GM-induced nephrotoxicity may come from both peroxynitrite and Haber-Weiss reaction. Interestingly, the OH scavenger dimethylthiourea also attenuates GM-induced *in vivo* lipid peroxidation [32] indicating the role of OH in lipid peroxidation.

The increase in $O_2^-$ and NO production in GM-induced nephrotoxicity may lead to peroxynitrite formation and could explain the increase in nitrosative stress in these animals.

We found that SAMC has *in vitro* scavenging properties; particularly we found that SAMC scavenges OH and $^1O_2$ (Fig. 1). These properties could be involved in the ability of SAMC to decrease lipid peroxidation which we evaluated by measuring 4-HNE. This is an α,β unsaturated aldehyde commonly used as a marker of lipid peroxidation due to it is produced in the peroxidative metabolism of arachidonic or linoleic acids [29]. HNE rapidly modifies proteins on several amino acids residues leading to the loss of protein functions. Antibodies against 4-HNE have been successfully used for the immunodetection of lipid peroxidation in kidney sections [2,29]. Interestingly, SAMC was also able to decrease 3-NT immunostaing *in vivo* suggesting that SAMC itself (or some of the metabolites) is able to scavenge *in vivo* peroxynitrite and/or another RNS involved in protein nitration. Immunohistochemical detection of 3-NT is a useful marker of nitrosative stress [2,7], since it has been shown that this compound is formed by the reaction of RNS with proteins [reviewed in [40]]. Surprisingly, SAMC was unable to scavenge $O_2^-$ and $H_2O_2$. SAMC was also able to prevent the increase in protein oxidation (measured by the increase in protein carbonyl content). Our data suggest that SAMC could be one of the compounds of AGE that may contribute to the ability of this extract to ameliorate GM-induced nephrotoxicity [10]. In a previous paper we found that S-allylcysteine, another compound found in AGE and structurally related to SAMC, also is able to prevent GM-induced nephrotoxicity [43].

The *in vivo* antioxidant ability of SAMC is associated with the improvement in GM-induced glomerular and tubular alterations. GM induces necrosis of proximal tubular cells but the structure of glomeruli is not altered. Vasoconstriction induced by ROS is involved in the decrease in glomerular filtration rate and ROS and RNS produce cellular injury and necrosis via several mechanisms, including lipoperoxidation and protein modification. The above information may explain why antioxidants, and SAMC in particular, are able to prevent GM-induced glomerular and tubular dysfunction. In our rats SAMC was able to ameliorate the decrease in creatinine clearance and blood GPx as well as the enhancement in BUN and in the urinary excretion of both total protein and NAG. SAMC also was able to ameliorate the histological damage. All the above

data clearly indicate the ability of SAMC to ameliorate GM-induced renal damage. Interestingly, SAMC was unable to prevent the increase in urinary volume induced by GM, this could be explained, in all probability, by the fact that the histological damage still is present in GM+SAMC rats (38% of damaged tubular area). This indicates that the amelioration in the histological damage is not enough to attenuate the increase in urinary volume.

The ameliorative effect of SAMC was not due to the enhancement of endogenous antioxidant defenses. It was found that SAMC itself was unable to modify the activity of Mn-SOD, Cu,Zn-SOD, GPx and GR but it decreased CAT activity (Table 2). SAMC was able to prevent the decrease in the antioxidant enzymes Mn-SOD, GPx and GR which also could contribute to the protective effect of SAMC.

## Conclusions

The protective effect of SAMC on GM-induced nephrotoxicity was associated with the decrease in oxidative and nitrosative stress *in vivo* and the preservation of Mn-SOD, GPx, and GR activities in renal cortex. Our data support that SAMC is one of the compounds of AGE with antioxidant properties *in vitro* and *in vivo*. The ability of SAMC to scavenge OH and $^1O_2$ may explain, at least in part, the *in vivo* antioxidant properties of SAMC. The potential protective effect of SAMC in humans receiving GM merits study.

## Methods

### *Reagents*

S-allylmercaptocysteine (SAMC, Lot # 020328) was kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan). GM was from Schering-Plough (Mexico City, Mexico). Rabbit anti-3-nitro-L-tyrosine (3-NT) polyclonal antibodies (Catalogue #06-284) were from Upstate (Lake Placid, NY, USA). Mouse anti-4-hydroxy-2-nonenal (4-HNE) monoclonal antibodies (# Catalogue 24325) were from Oxis International Inc. (Portland, OR, USA). Goat anti-dinitrophenol (DNP) polyclonal antibodies (Catalogue # J06) were from Biomeda Corporation (Foster City, CA, USA). Anti-rabbit Ig horseradish peroxidase antibody (Catalogue # NA-934) and anti-mouse Ig horseradish peroxidase antibody (Catalogue # NIF-825) were purchased from Amersham Life Sciences (Buckinghamshire, England). Donkey anti-goat horseradish peroxidase antibody (Catalogue # SC2020) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Commercial kits to measure creatinine and urea were from Spinreact (Girona, Spain). Glutathione reductase, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 2,4-dinitrophenylhydrazine (DNPH), histidine, ascorbic acid, D-ribose, tetramethoxypropane, deferoxamine mesylate, thiobarbituric acid, xylenol orange, butylated hydroxytoluene, iron (III) chloride ($FeCl_3$), *N,N*-dimethyl-4-nitrosoaniline were from Sigma-Aldrich (St. Louis MO, USA). Ferrous ammonium sulfate and sodium hypochlorite were from JT Baker (Xalostoc, Edo. México, México). All other chemicals were reagent grade.

### In vitro *experiments*

#### *Hydroxyl radical assay*

The ability of SAMC to scavenge OH was conducted in the $Fe^{3+}$-EDTA-$H_2O_2$-deoxyribose system [44]. The system contained different amounts of SAMC, or an equivalent volume of distilled water for the control, 0.2 mM ascorbic acid, 0.2 mM $FeCl_3$, 0.208 mM EDTA, 1 mM $H_2O_2$, 0.56 mM deoxyribose, and 20 mM phosphate buffer (pH 7.4). Hydroxyl radicals were generated by incubating the mixture at 37°C for 60 min. The iron salt ($FeCl_3$) was mixed with EDTA before addition to the reaction mixture. The extent of deoxyribose degradation by the formed OH was measured directly in the aqueous phase by the thiobarbituric acid test [45].

#### *Singlet oxygen assay*

The production of $^1O_2$ by sodium hypochlorite (NaOCl) and $H_2O_2$ was determined by using a spectrophotometric method [46] with minor modifications in which *N,N*-dimethyl-*p*-nitrosoaniline was used as selective acceptor of $^1O_2$. The bleaching of *N,N*-dimethyl-*p*-nitrosoaniline was monitored spectrophotometrically at 440 nm. The assay mixture contained 45 mM sodium-phosphate buffer (pH 7.1), 10 mM histidine, 10 mM NaOCl, 10 mM $H_2O_2$ and 50 μM *N,N*-dimethyl-*p*-nitrosoaniline. The total volume reaction was 2.0 ml and incubated at 30°C for 40 min. The extent of $^1O_2$ production was determined by measuring the decrease in the absorbance of *N,N*-dimethyl-*p*-nitrosoaniline at 440 nm. The relative scavenging efficiency (% inhibition in production of $^1O_2$) was estimated from the difference in absorbance of *N,N*-dimethyl-*p*-nitrosoaniline with and without the addition of increasing amounts of SAMC.

#### *Superoxide anion assay*

Xanthine-xanthine oxidase system was used to determine the $O_2^-$ scavenging activity of SAMC. $O_2^-$ production and xanthine oxidase activity were measured as NBT reduction and uric acid production, respectively [47] using a DU-64O series Beckman spectrophotometer. Tubes without SAMC were taken as 100% of NBT reduction.

#### *Hydrogen peroxide assay*

A solution of 75 μM $H_2O_2$ was mixed with different concentrations of SAMC (1:1 v/v) and incubated for 3.5 h at room temperature. After this, $H_2O_2$ was measured by the method described by Long *et al*. [48]. Briefly, 9 volumes of 4.4 mM butylated hydroxytoluene in HPLC-grade methanol were mixed with 1 volume of 1 mM xylenol orange

and 2.56 mM ammonium ferrous sulfate in 0.25 M $H_2SO_4$ to give the "working" FOX reagent. Ninety microliters of the $H_2O_2$-SAMC solutions were pippeted in 1.5 mL Eppendorf tubes and mixed with 0.01 mL of HPLC-grade methanol immediately followed by the addition of 0.9 mL of FOX reagent, vortexed for 5 seconds and then incubated at room temperature for 10 minutes. The tubes were centrifuged for 15,000 × g for 10 minutes and absorbance at 560 nm was reading against methanol blank.

### In vivo *experiments*

#### *Experimental design*

Male Wistar rats (Harlan Teklad, México City, México) initially weighing 240–250 g were used. Experimental work was approved by CONACYT (#40009-M) and followed the guidelines of Norma Oficial Mexicana (NOM-ECOL-087-1995). All animals had free access to water and commercial rodent diet (Harlan Teklad, catalogue 2018S), and were randomly divided in four groups (n = 5 rats/group) as follows: (1) CT, injected subcutaneously (s.c.) with saline and intragastrically (i.g.) with carboxymethylcellulose 0.5%, (2) GM, treated s.c. with GM and i.g. with carboxymethylcellulose 0.5%; (3) SAMC, treated with SAMC; and (4) GM+SAMC, treated with GM and with SAMC. GM treated rats received 70 mg/Kg b.w./s.c./12 h for 4 days and SAMC treated rats received a single dose of 100 mg/Kg b.w./i.g. 24 h before the first dose of GM or saline, and 50 mg/kg b.w./i.g./12 h for 4 days alongside GM-treatment or saline [23]. SAMC was given 30 minutes before every GM injection. SAMC was suspended in 0.5% carboxymethylcellulose at a 10 mg/ml concentration. Rats received 5.0 ml/Kg/12 h (50 mg SAMC/Kg/12 h). The dose of SAMC was chosen from the previous work of Sumioka *et al*. [23]. During the study rats were maintained with a 12-h light:dark cycle in stainless steel metabolic cages to collect urine. On day 5, the animals were sacrificed by decapitation and blood was collected to obtain serum and measure creatinine, BUN and GPx activity. NAG and total protein were measured in 24-h urine. One kidney was removed to obtain cortex samples for histological and immunohistochemical studies and the other kidney was removed to obtain cortex and measure the activity of total SOD, Mn-SOD, CAT, GPx, and GR as well as the carbonyl content of proteins.

#### *Markers of glomerular and tubular damage*

The markers of glomerular damage, creatinine and urea, were measured using commercial kits. Creatinine clearance was calculated with the standard formula [49], and BUN was obtained by correcting the urea value by a 2.14 factor [50]. As markers of tubular damage, we measured urinary excretion of NAG and total protein and blood GPx activity. NAG activity was measured using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate [17], total protein was measured by a turbidimetric method [6], and blood GPx activity was measured using GR and NADPH in a coupled reaction [10].

#### *Histological analysis*

Thin slices of kidney tissue with cortex and medulla were fixed by immersion in buffered formalin (pH 7.4), dehydrated and embedded in paraffin. Sections (4 μm) were stained with hematoxilin and eosin (H&E) [7]. The histological profile of twenty proximal tubules randomly selected per rat (5 rats per experimental group) was recorded, using a Leica Qwin Image Analyzer (Cambridge, England). The percentage of tubular area with histopathological alterations like swelling, cytoplasmic vacuolization, desquamation or necrosis was obtained. The percentage of damaged area of GM and GM+SAMC groups was compared.

#### *Protein carbonyl content*

Protein carbonyl groups are relatively stable marker of protein oxidation by ROS [28]. Carbonyl moieties in kidney tissues from all experimental groups were assessed using both spectrophotometric and immunohistochemical detection of the formation of the stable dinitrophenyl (DNP) hydrazone product which is formed when protein carbonyl groups reacts with DNPH. The spectrophotometric method has been described previously [28] and the immunohistochemical method will be described in the next section.

#### *Immunohistochemical localization of 3-NT, DNP, and 4-HNE*

For immunohistochemistry, 4 μm sections were deparaffined with xylol and rehydrated with ethanol. Endogenous peroxidase was quenched/inhibited with 4.5% $H_2O_2$ in methanol by incubation for 1.5 h at room temperature. The sections used for DNP immunohistochemistry were incubated with 0.2% DNPH in 2 N HCl for 60 min at room temperature in absence of light and then were extensively washed. Nonspecific adsorption was minimized by leaving the sections in 3% bovine serum albumin in phosphate buffer saline for 30 min. Sections were incubated overnight with a 1:700 dilution of anti-3-NT antibody [7] or with a 1:500 dilution of anti-DNP antibody or with a 1:100 dilution of anti-4-HNE antibody. After extensive washing with phosphate buffer saline, the sections were incubated with a 1:500 dilution of a peroxidase conjugated anti-rabbit Ig antibody (for 3-NT) or with a 1:500 dilution of a peroxidase conjugated anti-goat Ig or anti-mouse IgG (for DNP and 4-HNE, respectively) for 1 h, and finally incubated with hydrogen peroxide-diaminobenzidine for 1 min. Sections were counterstained with hematoxilin (for 3-NT and 4-HNE) or with methyl green (for DNP) and observed under light microscopy. All the sections from the four studied groups were incubated under the same conditions with the same anti-

bodies concentration, and in the same running, so the immunostaining was comparable among the different experimental groups.

*Antioxidant enzymes in renal cortex*

Total SOD activity was assayed by a previously reported method using nitroblue tetrazolium (NBT) [17]. To measure Mn-SOD activity, Cu,Zn-SOD was inhibited with diethyldithiocarbamic acid (DDC) [17]. Cu,Zn-SOD activity was obtained by subtracting the activity of the DDC-treated samples from that of total SOD activity. GPx activity was assayed as previously described [17] and GR activity was assayed measuring the disappearance of NADPH [10]. CAT activity was assayed by a method based on the disappearance of $H_2O_2$ [6].

### *Statistics*

Results are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons. Non-paired *t*-test was used to compare the quantitative histological damage data using the software Prism 3.02 (GraphPad, San Diego, CA, USA). $P \leq 0.05$ was considered statistically significant.

## List of abbreviations

3-NT 3-nitrotyrosine

4-HNE 4-hydroxy-2-nonenal

AGE Aged garlic extract

BUN Blood urea nitrogen

GM Gentamicin

CAT Catalase

CT Control

Cu,Zn-SOD Copper,zinc-dependent superoxide dismutase

DDC Diethyldithiocarbamic acid

DNP Dinitrophenol

DNPH 2,4-dinitrophenylhydrazine

GPx Glutathione peroxidase

GR Glutathione reductase

HO Hydroxyl radical

$H_2O_2$ Hydrogen peroxide

Mn-SOD Manganese-dependent superoxide dismutase

NADPH β-nicotinamide adenine dinucleotide phosphate, reduced form

NAG N-acetyl-β-D-glucosaminidase

NBT Nitroblue tetrazolium

NO Nitric oxide

$^1O_2$ Singlet oxygen

$O_2^-$ Superoxide anion

ROS Reactive oxygen species

RNS Reactive nitrogen species

SAMC S-allylmercaptocysteine

SEM Standard error of mean

SOD Superoxide dismutase

## Authors' contributions

JPCH conceived, designed and coordinated the study and drafted the manuscript. PDM, YICH, NAMR, and MIS performed animal experimentation, biochemical determinations and light microscopy studies. DB performed the histological and immunohistochemical studies. LC and NAMR measured the activity of antioxidant enzymes. ONMC performed *in vitro* ROS scavenging assays of SAMC. RHP supported the light microscopy and immunohistochemical studies.

## Additional material

### Additional File 1

*Inability of SAMC to scavenge superoxide anions ($O_2^{\bullet-}$) (A) and hydrogen peroxide ($H_2O_2$) (B). Data are mean ± SEM.*n = *3–4. Scavenging activity of SAMC for superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide ($H_2O_2$) was tested in vitro according to the methods described in Material and Methods. In both cases we were unable to see scavenging activity of SAMC. The highest concentration of SAMC that we could use in these particular assays was 0.5 mM.*

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