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Effect of endogenous hydrogen sulfide inhibition on structural and functional renal disturbances induced by gentamicin

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Abstract

Animal models of gentamicin nephrotoxicity present acute tubular necrosis associated with inflammation, which can contribute to intensify the renal damage. Hydrogen sulfide (H₂S) is a signaling molecule involved in inflammation. We evaluated the effect of DL-propargylglycine (PAG), an inhibitor of endogenous H₂S formation, on the renal damage induced by gentamicin. Male Wistar rats (N = 8) were injected with 40 mg/kg gentamicin (*im*) twice a day for 9 days, some of them also received PAG (N = 8, 10 mg·kg⁻¹·day⁻¹, *ip*). Control rats (N = 6) were treated with saline or PAG only (N = 4). Twenty-four-hour urine samples were collected one day after the end of these treatments, blood samples were collected, the animals were sacrificed, and the kidneys were removed for quantification of H₂S formation and histological and immunohistochemical studies. Gentamicin-treated rats presented higher sodium and potassium fractional excretion, increased plasma creatinine [4.06 (3.00; 5.87) mg%] and urea levels, a greater number of macrophages/monocytes, and a higher score for tubular interstitial lesions [3.50 (3.00; 4.00)] in the renal cortex. These changes were associated with increased H₂S formation in the kidneys from gentamicin-treated rats (230.60 ± 38.62 µg·mg protein⁻¹·h⁻¹) compared to control (21.12 ± 1.63) and PAG (11.44 ± 3.08). Treatment with PAG reduced this increase (171.60 ± 18.34), the disturbances in plasma creatinine levels [2.20 (1.92; 4.60) mg%], macrophage infiltration, and score for tubular interstitial lesions [2.00 (2.00; 3.00)]. However, PAG did not interfere with the increase in fractional sodium excretion provoked by gentamicin. The protective effect of PAG on gentamicin nephrotoxicity was related, at least in part, to decreased H₂S formation.

Key words: Inflammation; Gentamicin nephrotoxicity; DL-propargylglycine; Hydrogen sulfide; Acute tubular necrosis

Introduction

The principal side effect of gentamicin is acute tubular necrosis with acute renal failure observed in about 20% of exposed patients and can reach 58% of the patients in intensive care units (1). Recovery of baseline renal function is slow and may be incomplete, probably because of permanent loss of nephrons. Animal models of gentamicin nephrotoxicity show acute tubular necrosis associated with inflammation in the renal cortex (2,3). The inflammatory process can contribute to intensifying the renal damage. Lymphocytes and macrophages present in the inflammatory process can mediate the injury through various mechanisms,

including generation of reactive oxygen species, nitric oxide, complement factors, and pro-inflammatory cytokines (4).

Hydrogen sulfide (H₂S) is an important signaling molecule involved in inflammation (5-7). This gas is synthesized in the body from L-cysteine by different pathways in which several enzymes are involved, such as: cystathionine-γ-lyase (CSE), cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase, cysteine aminotransferase, and cysteine lyase (6). Pretreatment of animals with DL-propargylglycine (PAG), a CSE inhibitor, reduced the formation of tissue H₂S and inflammation induced by

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lipopolysaccharide, decreased the activity of hepatic myeloperoxidase (a marker for neutrophil infiltration) and the tissue damage (5). In a recent study from our laboratory, we observed that treatment with PAG reduced the renal damage induced by cisplatin injection (8). This effect was related to the reduction of H₂S formation and the reduction of the inflammation in the kidneys of PAG+cisplatin-treated rats. H₂S also has anti-inflammatory activity depending on its rate of generation (6). It was observed that H₂S at physiological concentrations presents anti-inflammatory effects (9). The present study evaluated the effect of PAG on the renal damage induced by gentamicin, when administered 4 h after the initiation of gentamicin treatment. PAG is an irreversible inhibitor of CSE and, when administered to rodents, produces an almost complete inhibition of the activity of this enzyme (10), is well absorbed and rapidly crosses biological membranes (11).

Material and Methods

Animals and experimental protocols

A total of 26 male Wistar rats (200-250 g) were provided by the Animal House of the Ribeirão Preto Campus, University of São Paulo (Ribeirão Preto, SP, Brazil) and housed in polycarbonate cages (4 animals per cage) under standard room temperature (25°C), and on a 12-h light/dark cycle with free access to standard rat chow and water. Eight rats were injected with gentamicin (Schering-Plough S/A, Brazil, 40 mg/kg, *im*, twice a day, for 9 days, 8 received gentamicin+PAG for 9 days, 4 with PAG alone (Sigma, USA), and 6 control rats were treated with 0.15 M NaCl solution. PAG was injected for 9 days (10 mg·kg⁻¹·day⁻¹, *ip*) starting on day one of gentamicin treatment and the injections were performed 4 h after the first daily injection of gentamicin and 4 h before the second. The dose and time selected for gentamicin or PAG treatment were based on recent studies performed in our laboratory (3,8). One day after the end of these treatments, 24-h urine samples were collected in metabolic cages; blood samples were also collected and the animals were sacrificed by excess anesthesia and the kidneys removed for H₂S formation evaluation and histological and immunohistochemical studies. All experimental procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the Animal Experimentation Committee of Faculdade de Medicina de Ribeirão Preto, Universidade de Paulo, approved the study protocol.

Renal function studies

One day after the end of these treatments the rats of all groups were placed in metabolic cages and 24-h urine samples were collected to measure creatinine, sodium and potassium. Plasma samples were then collected and the kidneys removed. Plasma creatinine and blood urea nitrogen

were measured using commercial kits (Labtest Diagnostica S.A., Brazil), and sodium and potassium were determined in plasma and urine using flame photometry (model 262; Micronal, Brazil). Urine creatinine was measured in order to calculate fractional sodium and potassium excretion by dividing sodium or potassium clearance (U_{Na} or $U_K \times V / P_{Na}$ or P_K) by creatinine clearance ($U_{creat} \times V / P_{creat}$, where U_{Na} and U_K = urinary sodium and potassium concentrations; V = urinary volume/min; P_{Na} or P_K = plasma sodium and potassium concentrations and U_{creat} and P_{creat} = urinary and plasma creatinine concentrations).

Light microscopy studies

The kidneys were sectioned transversely, fixed in 4% paraformaldehyde, postfixed in Bouin's solution for 4-6 h, and processed for paraffin embedding. Histological sections (4- μ m thick) were stained with Masson's trichrome and examined under the light microscope. Tubulointerstitial damage was defined as tubular necrosis, inflammatory cell infiltrate, tubular lumen dilation, or tubular atrophy. Damage was scored on a scale of 0 to 4 [0 = normal; 0.5 = small focal areas; 1 = involvement of less than 10% of the cortex; 2 = 10-25% involvement of the cortex; 3 = 25-75% involvement of the cortex; 4 = extensive damage involving more than 75% of the cortex (12)].

Antibodies

The macrophage/monocyte infiltrations in renal tissue related to the inflammatory process were evaluated using a monoclonal antibody (ED1; Serotec, UK) that only reacts against antigens present in the cytoplasm of macrophages and monocytes. Tubular cell lesions were evaluated using a monoclonal antibody against vimentin (Dako, Denmark). Tubular cells only express vimentin when they are proliferating. Therefore, increased vimentin expression in tubular cells suggests recent tubular cell injury (13).

Immunohistochemical study

Kidney sections were submitted to immunohistochemical study (13). The sections were incubated for 1 h with 1/1000 monoclonal anti-ED1 or with 1/500 monoclonal anti-vimentin antibodies. The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, USA). The color reaction was developed with 3,3'-diaminobenzidine (DAB; Sigma). The sections were counterstained with methyl green, dehydrated and mounted. Negative controls were created by replacing the primary antibody with equivalent concentrations of normal mouse immunoglobulin G. For the evaluation of the immunoperoxidase stain for vimentin each grid field of renal cortex measuring 0.245 mm² was graded semiquantitatively, and the mean score per biopsy was calculated. Each score reflected mainly the changes in the extent rather than the intensity of staining and depended on the percentage of the grid field showing positive staining: 0 = absent staining

or less than 5% of stained area; I = 5 to 25%; II = >25 to 50%, III = >50 to 75%, and IV = >75%. Immunoperoxidase staining for ED1 was determined by counting the positive cells (infiltrating macrophages and monocytes) in the renal cortical tubulointerstitium by the examination of 30 grid fields, measuring 0.100 mm² each, and the mean counts per area of 0.245 mm² per kidney were calculated (13). The evaluation of renal histology and immunohistochemical studies were performed in a blind way by two different observers.

Assay of H₂S synthesis

H₂S biosynthesis was measured as described previously (14). Whole kidney cortical tissue from the rats were homogenized in 100 mM potassium phosphate buffer, pH 7.4, using a Polytron device. Each sample (200 µL, 50% weight/volume) containing 20 µL 10 mM L-cysteine, 20 µL 2 mM pyridoxal 5'-phosphate, and 30 µL PBS was incubated for 2 h at 37°C. Zinc acetate (1% (w/v); 100 µL) was then added to trap evolved H₂S, followed by trichloroacetic acid (10% (w/v); 100 µL) to precipitate protein and thus stop the reaction. After centrifugation, 50 µL 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate followed by 50 µL 30 mM FeCl₃ were then added to 50 µL supernatant, and absorbance at 670 nm was measured. The H₂S concentration of each sample was calculated against a calibration curve of 100-0.1 µg/mL NaHS. All reagents used in this assay were from Sigma.

Statistical analysis

Data concerning plasma urea and creatinine levels, sodium and potassium fractional excretion, and scores for tubulointerstitial lesions were analyzed statistically using the nonparametric Kruskal-Wallis test followed by the Dunn post-test. These data are reported as median and interquartile range (25-75% percentile). For data related to the other parameters studied we used analysis of variance with the multiple comparisons Newman-Keuls test. These

data are reported as means ± SEM. The level of statistical significance was set at $P < 0.05$.

Results

Gentamicin-treated rats presented increases in plasma creatinine and urea levels and in sodium and potassium fractional excretions compared to control. Treatment with PAG attenuated the increase in plasma creatinine and urea levels and in fractional potassium excretion provoked by gentamicin. However, PAG did not prevent the increase of fractional sodium excretion observed in gentamicin-treated rats. PAG only-treated rats did not present differences in any parameter of renal function studied compared to controls (Table 1).

Light microscopy study of the renal cortex of both groups of gentamicin-treated rats, sacrificed 2 days after the end of these treatments, showed the following morphological features: tubular cell necrosis, tubular lumen dilation, denudation of the tubular basement membrane, intraluminal casts, swelling/flattening of proximal tubular cells with brush border loss, diffuse interstitial edema, and interstitial inflammatory cell infiltrates. Glomerular morphology was unchanged. However, the score for tubulointerstitial lesions of renal cortex was higher in rats of the gentamicin group than in rats of the gentamicin+PAG group ($P < 0.01$; Figure 1A and B; Table 2). The immunohistochemical analysis for vimentin showed that gentamicin-treated rats presented high vimentin expression in the tubular cells of the renal cortex compared to controls, suggesting recent cell injury. This alteration was less intense in the animals of the gentamicin+PAG group ($P < 0.01$; Figure 1C and D; Table 2).

The data of the immunohistochemical study also showed that gentamicin-treated rats presented greater macrophage/monocyte numbers than the control group (Figure 1E and F), and this was associated with tubulointerstitial injury. Treatment with PAG, an inhibitor of H₂S formation, reduced macrophage infiltration ($P < 0.01$; Table 2).

Table 1. Parameters of renal function of saline-, PAG-, gentamicin-, and gentamicin+PAG-treated rats 2 days after the end of the treatment.

	Saline (N = 6)	PAG (N = 4)	Gentamicin (N = 8)	Gentamicin+PAG (N = 8)
P _{creat} (mg/mL)	0.49 (0.21; 0.61)	0.48 (0.46; 0.51)	4.06* (3.00; 5.87)	2.20 (1.92; 4.60)
BUN (mg/mL)	43.38 (34.10; 48.80)	46.30 (41.70; 56.10)	510.10* (400.80; 637.40)	263.80## (206.60; 476.20)
FE _{Na+} (%)	0.42 (0.32; 0.66)	0.66 (0.49; 0.70)	9.63* (7.34; 16.61)	8.60* (6.71; 20.87)
FE _{K+} (%)	44.37 (38.81; 52.80)	41.87 (36.96; 43.41)	181.10* (252.50; 234.40)	150.20 (136.70; 217.30)

Saline (0.15 M NaCl, *im*) and gentamicin (40 mg/kg, *im*) were injected twice a day for 9 days. PAG (10 mg·kg⁻¹·day⁻¹, *ip*) was injected for 9 days starting on day one of gentamicin treatment and the injections were performed 4 h after the first daily injection of gentamicin and 4 h before the second. PAG = DL-propargylglycine; P_{creat} = plasma creatinine; BUN = blood urea nitrogen; FE = fractional excretion. Data are reported as median and interquartile ranges (25-75% percentile). * $P < 0.01$ compared to saline; ## $P < 0.05$ compared to gentamicin (Kruskal-Wallis test followed by the Dunn post-test).

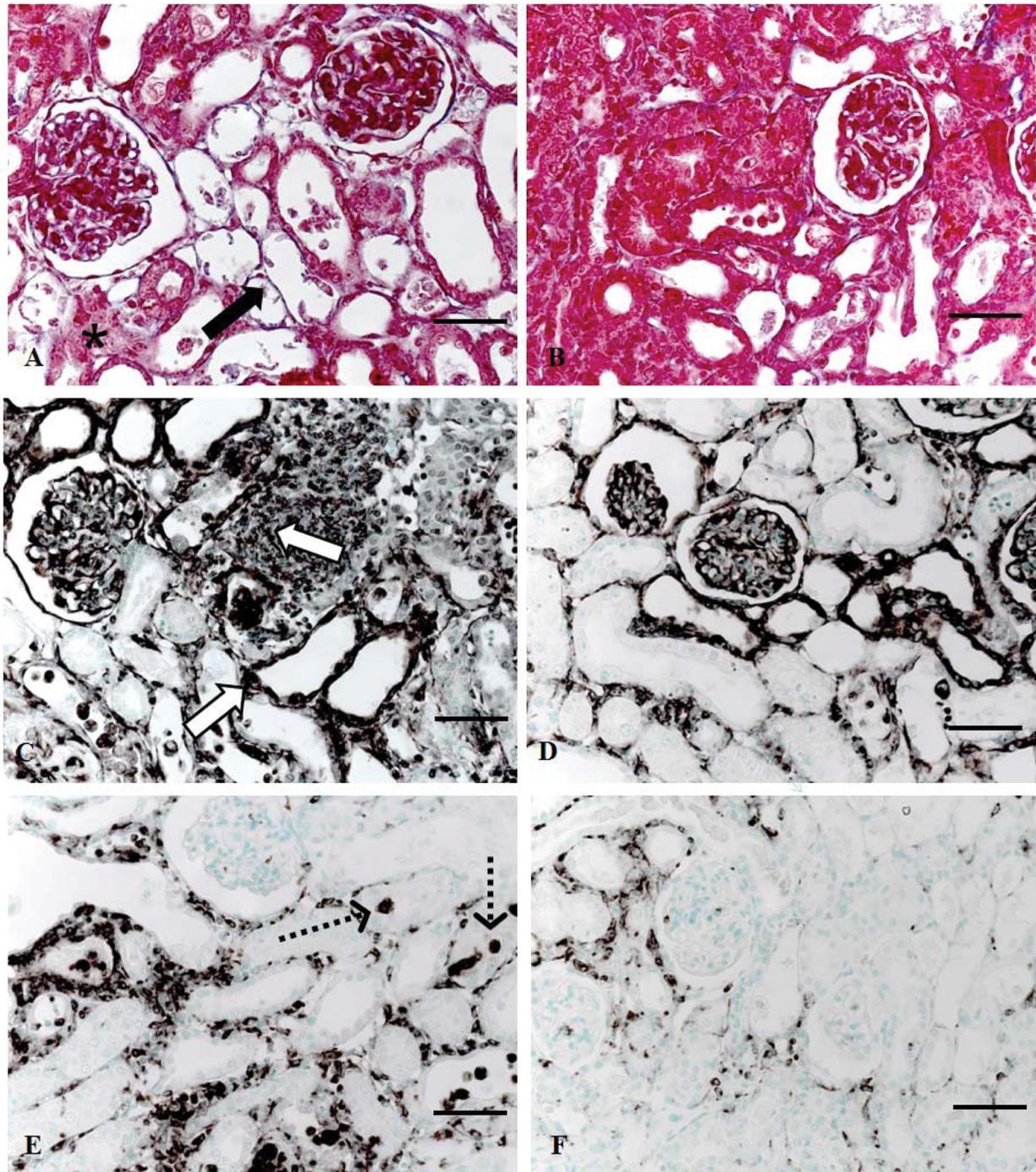


Figure 1. Masson's trichrome-stained histological sections (A and B) and immunolocalization of vimentin (C and D) and ED1⁺ cells (macrophages/monocytes; E and F) from the renal cortex of rats treated with gentamicin (A, C, E) or gentamicin+PAG (B, D, F), 2 days after the end of the treatment. Note the presence of tubular necrosis (black arrow) and the increase of relative interstitial area in A (asterisk). This alteration was less intense in B. Vimentin expression (white arrows) and the number of ED1⁺ cells (dotted arrows) were higher in C and E than in D and F. Magnification bars = 20 μ m.

Table 2. Score for tubulointerstitial lesions (TIL), vimentin and number of ED1⁺ cells (macrophages/monocytes) in the renal cortex of saline-, PAG-, gentamicin-, and gentamicin+PAG-treated rats two days after the end of treatment.

	Saline (N = 6)	PAG (N = 4)	Gentamicin (N = 8)	Gentamicin+PAG (N = 8)
TIL	0	0	3.50* (3.00; 4.00)	2.00 [#] * (2.00; 3.00)
Vimentin	0.10 (0.06; 0.12)	0.13 (0.004; 0.200)	2.89* (2.84; 3.43)	1.84 [#] * (1.69; 2.42)
ED1 ⁺ cells	4.94 ± 0.61	6.66 ± 0.41	154.30* ± 7.67	90.29 [#] * ± 8.68

Data are reported as median and interquartile range (25-75% percentile) for TIL and vimentin (data compared by the Kruskal-Wallis test followed by the Dunn post-test) or mean ± SEM for ED1⁺ cells (data compared using analysis of variance with the multiple comparisons Newman-Keuls test). Drug treatments are given in the legend to Table 1. PAG = DL-propargylglycine. *P < 0.01 compared to saline; [#]P < 0.01 compared to gentamicin.

A significant increase in H₂S formation ($\mu\text{g}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$) was observed in the kidneys from gentamicin-treated rats (230.60 ± 38.62) compared to control (21.12 ± 1.63) and PAG (11.44 ± 3.08) ($P < 0.01$). Treatment with PAG attenuated the increase of H₂S provoked by gentamicin injection (171.60 ± 18.34 ; $P < 0.05$) by 25%.

Discussion

Experimental and clinical studies have shown that gentamicin treatment can provoke acute tubular necrosis with acute renal failure (1). Animal models of gentamicin nephrotoxicity show acute tubular necrosis associated with inflammation that contributes to intensifying the renal damage (2,3). In previous studies, we observed increased renal expression of cytokines such as transforming growth factor-beta (TGF- β), endothelin and angiotensin II in the renal cortex of rats sacrificed five days after the cessation of gentamicin treatment, which was related to renal functional and structural disturbances presented by these animals (2). These rats also presented activation of nuclear factor κB (NF- κB) and macrophage infiltration in the renal cortex (3).

The results of the present study showed that rats injected with gentamicin for 9 days and sacrificed two days after the end of this treatment presented higher plasma creatinine and urea levels, increased fractional sodium and potassium excretions, as well as higher numbers of macrophages/monocytes, tubular interstitial lesions and increased vimentin expression in tubular cells of the renal cortex compared to control. These changes were associated with an 11-fold increase of H₂S. The macrophage infiltration in the interstitial area from the renal cortex of gentamicin-treated rats was intense and diffuse. Macrophages can be involved in the inflammatory process that can progress to fibrosis in gentamicin nephrotoxicity (2,3). They are able to release peptides such as TGF- β , interleukin-1, endothelin, and angiotensin II (15). Treatment with PAG, an inhibitor of H₂S formation, reduced the macrophage infiltration and the

tubulointerstitial lesions, as well as the increases in plasma urea and creatinine levels provoked by gentamicin injection. PAG did not interfere with the higher fractional sodium excretion in gentamicin-treated rats. Although the tubular cell lesions were less intense in the animals treated with gentamicin plus PAG than in those injected with gentamicin alone, the renal protection conferred by PAG was related to the decrease in the inflammatory process, which also interfered with renal hemodynamics and glomerular filtration rate. Several mediators released by the inflammatory cells such as angiotensin II and endothelin can provoke an increase in renal arteriole resistance, leading to a decrease in renal blood flow (2). Therefore, this protective effect of PAG on renal hemodynamics may be more effective than on the tubular cell lesions. In addition, although PAG provoked a 25% decrease in the higher levels of H₂S formation induced by gentamicin treatment in rats, the H₂S levels were still higher in the renal cortex of the animals treated with gentamicin+PAG compared to control.

The increased biosynthesis of H₂S has been demonstrated in several animal models of inflammatory disease (septic/endotoxic and hemorrhagic shock, pancreatitis and carrageenan-induced hind paw edema in rats) and the inhibition of H₂S formation reduces the inflammation in these cases (5,16). However, the exact role of this gas on the inflammatory process is not known. In a study from our laboratory, we found that the role of H₂S in the inflammatory process cannot be direct, since the incubation of tubular epithelial cells with NaHS (a donor of H⁺ and S²⁻ for the synthesis of H₂S) did not modify cell viability (8). H₂S can induce neutrophil adhesion and locomotion during the inflammatory response by a mechanism dependent on ATP-sensitive potassium channels (7). Neutrophils release cytotoxic and pro-inflammatory mediators such as arachidonic acid metabolites, cytokines/chemokines, oxygen- and nitrogen-derived free radicals that have potent chemotactic effects leading to macrophage infiltration and kidney injury (17). H₂S can inhibit or activate NF- κB depending on the stage of inflammation. NF- κB activation in the early stages

of inflammation can lead to increased expression of several inflammatory substances such as cytokines, chemokines and adhesion molecules, intensifying the inflammatory process (7). Zhang et al. (18) observed that H₂S may induce an increase in tissue levels of adhesion molecules and promote leukocyte-endothelium interaction in sepsis through a mechanism involving the activation of NF-κB.

In another study, Zhang et al. (19) observed that intraperitoneal administration of PAG led to a peak serum concentration within 1 to 2 h, followed by rapid excretion in urine. Kodama et al. (20) showed that intraperitoneal PAG injection in rats resulted in almost complete inhibition of liver CSE activity (without any effect on CBS) within 120 min.

Our data show that treatment with PAG, an inhibitor of

H₂S formation, reduces disturbances in renal function as well as macrophages infiltration in renal cortex and tubule interstitial lesions provoked by gentamicin. The protective effect of PAG on gentamicin nephrotoxicity was associated with a reduction of H₂S formation.

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