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Hydrogen sulfide protects from acute kidney injury via attenuating inflammation activated by necroptosis in dogs

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ABSTRACT

Background: The treatment of acute kidney injury (AKI), a common disease in dogs, is limited. Therefore, an effective method to prevent AKI in veterinary clinics is particularly crucial.
Objectives: Hydrogen sulfide (H_2S) is the third gaseous signal molecule involved in various physiological functions of the body. The present study investigated the effect of H_2S on cisplatin-induced AKI and the involved mechanisms in dogs.
Methods: Cisplatin-injected dogs developed AKI symptoms as indicated by renal dysfunction and pathological changes. In the H_2S-treated group, 50 mM sodium hydrosulfide (NaHS) solution was injected at 1 mg/kg/h for 30 min before cisplatin injection. After 72 h, tissue and blood samples were collected immediately. We performed biochemical tests, optical microscopy studies, analysis with test kits, quantitative reverse-transcription polymerase chain reaction, and western blot analysis.
Results: The study results demonstrated that cisplatin injection increased necroptosis and regulated the corresponding protein expression of receptor interacting protein kinase (RIPK) 1, RIPK3, and poly ADP-ribose polymerase 1; furthermore, it activated the expressions of inflammatory factors, including tumor necrosis factor-alpha, nuclear factor kappa B, and interleukin-1β, in canine kidney tissues. Moreover, cisplatin triggered oxidative stress and affected energy metabolism. Conversely, an injection of NaHS solution considerably reduced the aforementioned changes.
Conclusions: In conclusion, H_2S protects the kidney from cisplatin-induced AKI through the mitigation of necroptosis and inflammation. These findings provide new and valuable clues for the treatment of canine AKI and are of great significance for AKI prevention in veterinary clinics.

Keywords: Acute kidney injury; hydrogen sulfide; necroptosis; inflammation; dogs

INTRODUCTION

Acute kidney injury (AKI), an independent risk factor for mortality, is a clinical syndrome characterized by a rapid decline of renal function in humans. As one of the most common complications in hospitalized patients, AKI increases the risk of death by 10–15 times and has a mortality rate of 50%, which accounts for approximately 2 million deaths per year

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Conflict of Interest
The authors declare no conflicts of interest.

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worldwide [1,2]. In dogs, acute kidney injury is a common disease reported in veterinary clinics, and effective prevention and timely treatment of this disease can prevent substantial kidney damage. A survey revealed that 19% of the AKI reported are induced by drug nephrotoxicity in humans [3]. Dialysis is crucial for the removal of toxic byproducts of drug metabolism. In addition, a report stated that the mortality rate of AKI is 47%–61% in dogs [4]. Therefore, the development of an effective method to prevent canine AKI is crucial.

Necroptosis is one of the cell death modes in AKI; it is a type of programmed cell death mediated by receptor interacting protein kinase (RIPK) signaling [5]. RIPK1 is a key factor in necroptosis initiation; it combines with tumor necrosis factor receptor (TNFR) 1, TNFR1-associated death domain (TRADD) protein, and TNFR-associated factor (TRAF)-2 through the death domain to form complex I, which can induce necroptosis through the formation of the RIPK1/RIPK3/mixed lineage kinase domain-like necrosome in the absence of caspase-8 (Cas8) [6,7]. In recent years, many studies have revealed that necroptosis is associated with inflammation. For instance, Welz et al. [8] demonstrated that RIPK3 gene deficiency prevented inflammation and cell death in both the small intestine and colon of mice. Murakami et al. [9] revealed that programmed necrosis promoted inflammation through the regulation of the release of intracellular damage-associated molecular patterns in mice with retinal degeneration. Additionally, RIPK3 can activate glutamate-ammonia ligase, thereby increasing glutamate decomposition, and mitochondrial glutamate catabolism leads to local free ammonia accumulation and increases reactive oxygen species (ROS) expression [9,10]. Thus, necroptosis may be associated with oxidative stress.

Hydrogen sulfide (H_2S) is the third endogenous gaseous signal molecule after nitric oxide and carbon monoxide, which plays a crucial role in various tissues in both health and disease [11]. H_2S was initially identified as a harmful exogenous gas with a pungent smell that can damage various tissues and organs of the body [12]. In 1996, Abe and Kimura [13] discovered that H_2S can be produced by a series of enzymatic reactions in mammals; since then, the physiological function of H_2S has been gradually identified. In general, H_2S is synthesized from L-cysteine by three enzymes: cystathionine-β-synthase, cystathionine-β-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase [14]. These three enzymes are widely distributed in the cardio-cerebrovascular system, liver, and kidney, as well as in the cells of other tissues. Several studies have reported that H_2S plays a crucial role in inflammation. For example, H_2S can induce neutrophil apoptosis to reduce inflammation [15]. H_2S administration to rats with colitis downregulates the expression of proinflammation cytokine tumor necrosis factor-alpha (TNF-α), whereas the inhibition of H_2S synthesis in healthy rats induces inflammation in the small intestine and colon [16]. Furthermore, Chen et al. [17] showed that exogenous administration of sodium hydrosulfide (NaHS) can alleviate airway inflammation. In addition, some recent reports have highlighted that H_2S has significant antioxidant properties, which can upregulate the expression of key antioxidant enzymes and remove ROS [18,19]. King et al. [20] reported that after CSE knockout, the oxidative stress level in mice with myocardial infarction increased and myocardial injury aggravated, both of which were alleviated by exogenous H_2S.

Alleviation and prevention of AKI pathogenesis are of great significance in the veterinary field. In this study, we developed a canine AKI model with cisplatin, examined whether H_2S attenuates cisplatin nephrotoxicity, and explored the mechanism by which H_2S protects the kidney from cisplatin nephrotoxicity. To our knowledge, studies on cisplatin-induced AKI have focused on mice, and few reports are available regarding AKI in dogs and the effect of

H_2S on AKI. This study revealed a possibility of H_2S alleviating cisplatin-induced AKI in dogs, which will provide more possibilities for clinically reducing the side effects of drugs.

MATERIALS AND METHODS

Preparation of animals

All procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11). In total, 24 adult male beagles (8–12 kg) were divided randomly into three groups: control group (C), hydrogen sulfide + cisplatin group (H+cis), and cisplatin group (cis) (n = 8 per group, six were used for the experiments, and the remaining two were on standby for any unexpected condition). The laboratory staff cleaned the kennel regularly to ensure a healthy environment; the kennel temperature was controlled at 18°C–26°C. All dogs had access to standard food and water ad libitum during the study. Dogs in the cis group were injected with 5 mg/kg body weight cisplatin [21], and dogs in the C group were injected with an equal volume of saline. In the H+cis group, dogs were injected with 50 mM NaHS solution (1 mg/kg/h) 30 min before cisplatin injection (5 mg/kg). Dogs were anesthetized 72 h after the cisplatin injection. Blood and a small part of the left kidney tissues were quickly collected. Blood samples were collected for blood urea nitrogen (Bun) and serum creatinine (Scr) measurement. A portion of the collected kidney tissue was quickly removed and fixed in 10% phosphate-buffered formalin for hematoxylin and eosin (H&E) staining; the remaining tissue was quickly removed and frozen in liquid nitrogen and then stored at −80°C.

Serum analysis

The Bun and Scr levels of the tissues were evaluated using a UniCel DxC800 Synchron chemistry system (Beckman, USA). The renal injury model was considered to be established when the Bun and Scr levels of the cis group increased by twice as much as those of the C group.

Histopathological examination

The canine left kidney tissues were rapidly fixed in 10% formaldehyde for at least 24 h and then embedded in paraffin for microscopic examination. From the prepared paraffin blocks, 5-μm-thick sections were obtained and stained with H&E for light microscopic observation.

Detection of antioxidant levels

The kidney tissues were homogenized in physiological saline (1:10 w/v) with a glass Teflon homogenizer (Heidolph SO1 10R2RO). The homogenate was centrifuged at 700 × g for 30 min at 4°C to obtain the supernatant to measure the activities of superoxide (SOD) and catalase (CAT) as well as malondialdehyde (MDA) levels by using detection kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols.

Detection of ATPase

The activities of Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, and Ca^{2+}-ATPase were determined by using 10% tissue homogenates with appropriate assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. Inorganic phosphorus produced during the conversion of adenosine triphosphate to adenosine diphosphate was quantified using the molybdenum blue spectrophotometric method at 660 nm and expressed as U/mg.prot. When one type of ATPase was tested, the inhibitors of the other types of ATPase were added to depress the hydrolysis of phosphate radicals.

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Quantitative reverse-transcription polymerase chain reaction (qPCR) analysis

Total RNA from canine kidney tissues was extracted using TRIzol reagent according to the manufacturer's protocol. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm (Gene Quant 1300/100; General Electric Company, USA). qPCR was performed on a Light Cycler 480 System (Roche, Switzerland) after reverse transcription by using the fast qPCR kit (RR047A; Takara, Japan). All of the primers (**Table 1**) were designed using Premier Software (PREMIER Biosoft International, USA) for qPCR. The relative messenger RNA (mRNA) level was calculated according to the method of $2^{-\Delta\Delta Ct}$, and gene-specific efficiencies were normalized to the mean mRNA expressions of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunohistochemistry staining

The kidney sections were treated with 0.01 M sodium citrate buffer (pH 6.0) by using a microwave-based antigen retrieval technique for 20 min at 95°C, followed by 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Subsequently, they were incubated with RIPK1 (1:500; Bioss, China) and RIPK3 (1:500; Bioss) antibodies for 24 h at 4°C and secondary antibodies for 30 min at 37°C. After staining the slides with 3,3'-diaminobenzidine, they were observed under a microscope.

Western blot analysis

The protein samples were separated using 8%, 10%, and 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes (Cat# ISEQ. 00010, LOT# R6PA4145H; Merck Millipore, USA). The membranes were blocked with 5% skim milk for 3 h at 37°C and were incubated for 14 h at 4°C with the following diluted primary antibodies: pyruvate kinase (PK; 1:1,000; Wanlei, China), uncoupling protein 1 (UCP1; 1:1,500; Wanlei), succinate dehydrogenase (SDH; 1:500; Bioss), pyruvate dehydrogenase complex (PDHX; 1:500; Affinity, USA), lactate dehydrogenase (LDH; 1:1,000; Wanlei), poly ADP-ribose polymerase 1 (PARP1; 1:500; Proteintech, China), Cas8 (1:1,000; CST, USA), nuclear factor kappa B (NF-κB; 1:500; Wanlei), interleukin-1β (IL-1β; 1:1,000; Wanlei), and TNF-α (1:500; Wanlei). After washing thrice for 15 min each with

Table 1. mRNAs primer sequences

Gene	Forward 5' to 3'	Reverse 5' to 3'
PK	GACCGGCGGGCTACCTGAG	GCTGCTGCTGCTGGAAGAAGG
UCP1	AGAGGCAGGTGGGCTTCGC	GCTGCTCCTCCCGTCATTACAC
SDH	GGCGAGTCTCTGGAGGCTGAG	GGCTGGTCCTGGAGAAATGCTG
PDHX	CCTGGAGGCTGGGCTGTGAC	GCCGCTGCGTGCTGTGAAG
LDH	CGCTGCTGGCTACAACCTCAC	GCTGCCTGGACACTGGGAAAC
RIPK1	AGTGCCGGAGACCAACCTACTG	GGCTGAACTCATTCCACCAACCTC
RIPK3	TGGTGTCTGGAGTGGAGAGCTATG	CTGCTGTGCGGTCTGAACTGTC
PARP1	GGCTCTGATGACAGCAGCAAGG	TTCCTGATGGTCTCGGCTTCCTC
Cas8	TGGCTCTGATGGGCAGGAAGC	GGGCTTGCCTGCAAGGGAAG
NF-κB	CCTCTCGCTGCCCCTCTCATC	AGGTCTCCACGCCGCTGTC
TAK1	GCCATCATCCGCAACCTCATCC	AGCAGTCCACAGCCCTCATCC
TAB2	GGAAGCAGGACTCTAACGCACAG	GCCTTGAGGAACTTGAGCTGGTG
TAB3	GTTGTGGCTGCTACTCCGAACTAC	ATGGTTGTTGAGGTGGCTGTGAAG
IL-1β	TGTGAAGTGCTGCTGCCAAGAC	ACAGAGCTGGTGGGAGACTTGC
TNF-α	GGCGTGGAGCTGACAGACAAC	GACGGCGAAGCGGCTGATG
GAPDH	CAAGGCTGTGGGCAAGGTCATC	TTCTCCAGGCGGCAGGTCAG

mRNA, messenger RNA; PK, pyruvate kinase; UCP, uncoupling protein; SDH, succinate dehydrogenase; PDHX, pyruvate dehydrogenase complex; LDH, lactate dehydrogenase; RIPK, receptor interacting protein kinase; PARP, poly ADP-ribose polymerase; Cas8, caspase-8; NF-κB, nuclear factor kappa B; TAK, transforming growth factor-β activated kinase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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phosphate-buffered saline with Tween 20, the membranes were incubated for 2 h at 37°C with peroxidase-conjugated secondary antibodies against rabbit IgG (Cat# sc-2357, RRID: AB_628497; Santa Cruz Biotechnology, Argentina). After washing three times by PBST for 15 min each again, the bound antibodies were visualized through chemiluminescence by using the ECL-plus reagent (GE Healthcare, UK). The GAPDH content was analyzed as the loading control by using a rabbit polyclonal antibody.

Statistical analysis

Statistical analyses of all data were performed using GraphPad Prism (version 8.0; GraphPad Software Inc., USA). Significant values ($p < 0.05$) were obtained through a one-way analysis of variance. All data displayed normal distribution and passed the test for equal variance. The data are expressed as the mean ±SD, and the differences were considered to be significant if $p < 0.05$.

RESULTS

H_2S attenuates cisplatin-induced renal injury in dogs

As shown in **Supplementary Fig. 1**, after injection of NaHS solution, the levels of Bun and Scr in dogs were not significantly different from those in the control group, indicating that injection of NaHS solution had no effect on the kidneys of dogs. Besides, the content of Bun and Scr increased significantly after cisplatin treatment ($p < 0.01$), whereas H_2S had the opposite effect (**Fig. 1A and B**) In addition, we observed canine kidney tissues stained with H&E in the C, H+cis, and cis groups. Histopathological changes in renal tissues are presented in **Fig. 1C**. The kidney tissues in the C group displayed normal morphologies. However, some features indicating renal pathological damage were observed in the cis group; after cisplatin administration, the canine kidney tissue showed degeneration of renal tubular epithelial cells (blue arrow) and amyloidosis (yellow arrow). In addition, numerous inflammatory cells infiltrated the kidney tissue (red arrow). As expected, in the H+cis group, renal pathological damage was relieved, but some changes remained compared with the C group, including a small amount of inflammatory cell infiltration (red arrow).

Antioxidant capacity in canine kidney tissues

The results of antioxidant activity of canine kidney tissues were as follows. Compared with the C group, the activities of SOD and CAT in the cis group significantly decreased ($p < 0.01$); however, after the addition of H_2S, the aforementioned antioxidant enzyme activities were restored (**Fig. 2A and B**). In addition, no significant difference was observed between the C and H+cis groups in terms of MDA levels ($p > 0.05$), whereas MDA levels were upregulated in the cis group compared with the C and H+cis groups ($p < 0.01$; **Fig. 2C**).

ATPase activities in canine kidney tissues

All ATPase activities weakened significantly after cisplatin treatment ($p < 0.01$; **Fig. 3**). Notably, the Ca^{2+}-ATPase activity in the cis group decreased the most by approximately 31.5% (**Fig. 3C**). Furthermore, compared with the cis group, the activities of Ca^{2+}-Mg^{2+}-ATPase and Ca^{2+}-ATPase increased significantly ($p < 0.05$; **Fig. 3B and C**), and Na^{+}-K^{+}-ATPase activity increased nonsignificantly ($p > 0.05$) (**Fig. 3A**) in the H+cis group.

Expression levels of energy metabolism–related genes in canine kidney

The expression levels of PK, UCP1, SDH, PDHX, and LDH markedly decreased ($p < 0.01$) after cisplatin treatment (**Fig. 4**). Moreover, the expression of all energy metabolism–related genes in

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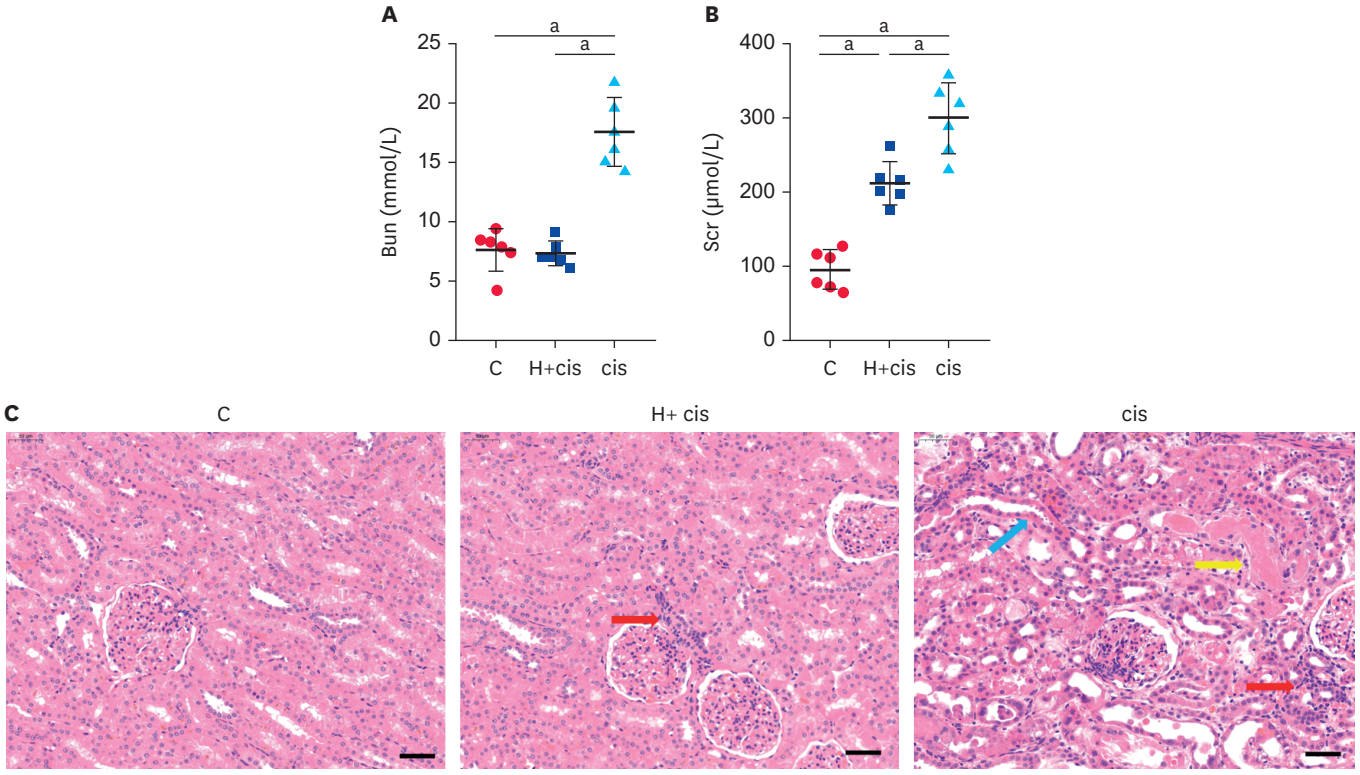

Fig. 1. H_2S attenuated cisplatin-induced renal injury in dogs. (A) The result of Bun. (B) The result of Scr. (C) Histopathological changes for kidney tissue in dogs. Data are expressed as the mean ± SD (n = 6). Scale bars = 50 μm. Red arrow: inflammatory cell infiltration; blue arrow: degeneration of renal tubular epithelial cells; yellow arrow: amyloidosis in canine kidney.
C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group; Bun, blood urea nitrogen; Scr, serum creatinine.
[a]$p < 0.01$ presents a significant difference.

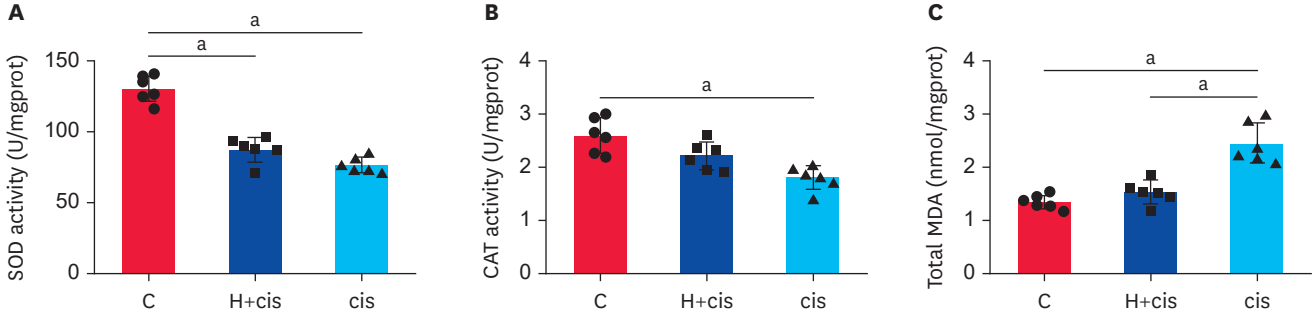

Fig. 2. The antioxidant capacity in canine kidney. (A) The activity of SOD. (B) The activity of CAT. (C) The content of MDA. Data are expressed as the mean ± SD (n = 6).
C, control group; H+cis, hydrogen sulfide and cisplatin group, cis, cisplatin group; SOD, superoxide; CAT, catalase; MDA, malondialdehyde.
[a]$p < 0.01$ presented a significant difference.

the H+cis group increased significantly compared with that in the cis group ($p < 0.01$; **Fig. 4A-F**). Among them, the relative expression of PK and the protein expression of LDH in the H+cis group was slightly higher than that in the C group (**Fig. 4A and C**).

Relative expressions of necroptosis-related genes in canine kidney tissues

The effect of cisplatin on the relative expression of necroptosis-related genes and the role of H_2S in canine kidney tissues are shown in **Fig. 5**. Cisplatin treatment significantly increased the mRNA and protein levels of necrosis genes, including RIPK1 and RIPK3, whereas H_2S pretreatment markedly reduced the levels of RIPK1 and RIPK3 ($p < 0.01$) (**Fig. 5A-D**).

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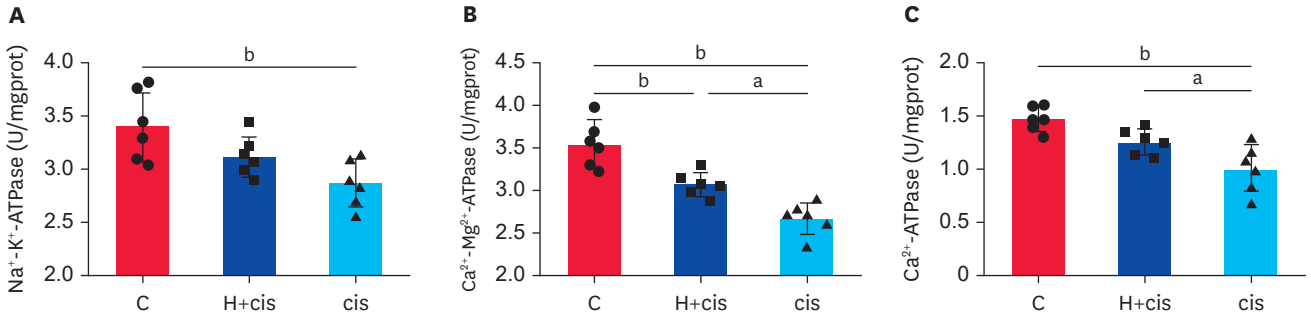

Fig. 3. ATPase activity in canine kidney. (A) The activity of Na^{+}-K^{+}-ATPase. (B) The activity of Ca^{2+}-Mg^{2+}-ATPase. (C) The activity of Ca^{2+}-ATPase. Data are expressed as the mean ± SD (n = 6).
C, control group; H+cis, hydrogen sulfide and cisplatin group, cis, cisplatin group.
[a]$p < 0.05$, [b]$p < 0.01$ presented a significant difference.

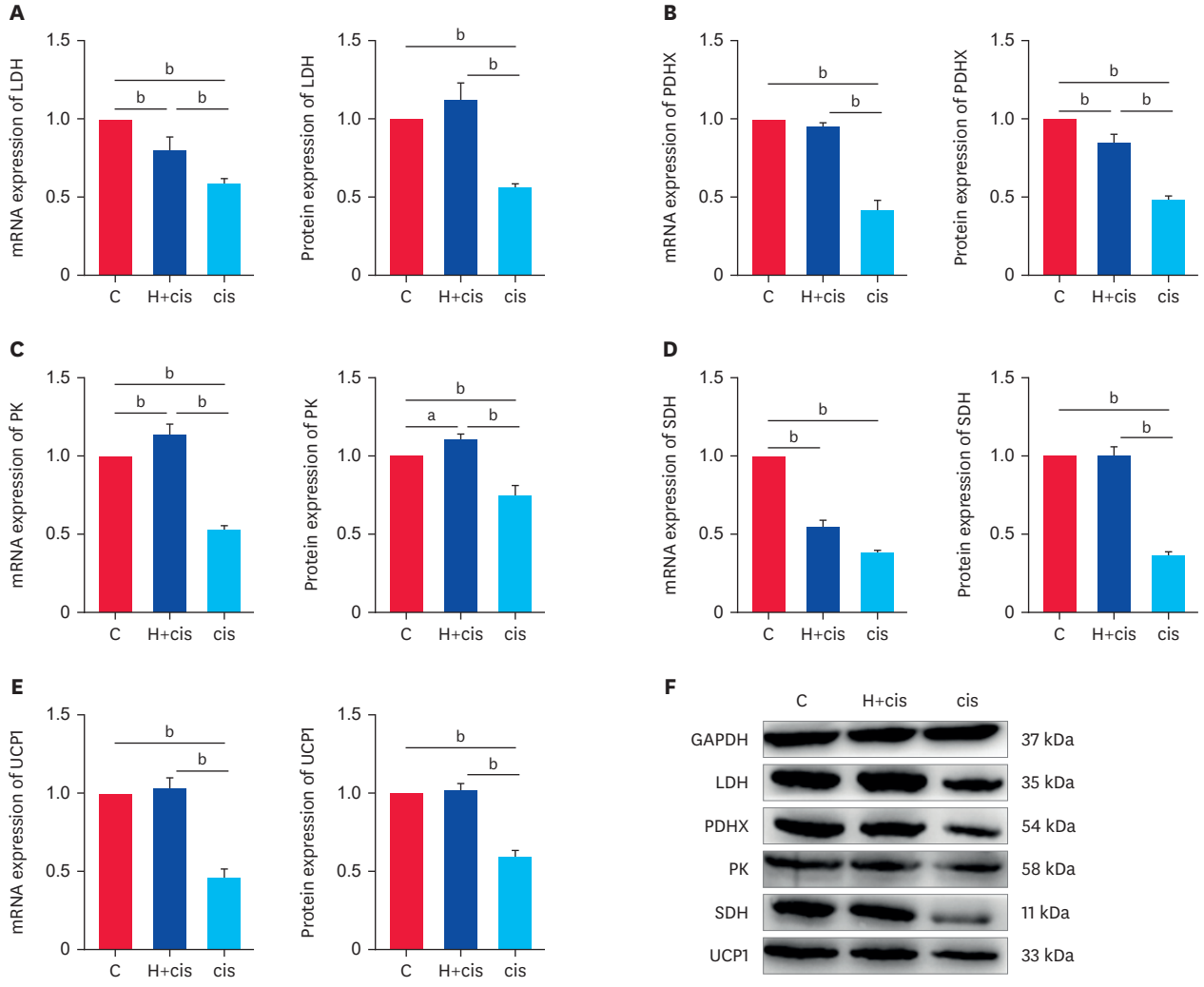

Fig. 4. The expression levels of energy metabolism-related genes in canine kidney. (A) The mRNA and protein expression of LDH. (B) The mRNA and protein expression of PDHX. (C) The mRNA and protein expression of PK. (D) The mRNA and protein expression of SDH. (E) The mRNA and protein expression of UCP1. (F) Western blotting for LDH, PDHX, PK, SDH and UCP1 in canine kidney. GAPDH was selected as the reference of mRNA and protein expressions. Data are represented as the mean ± SD (n = 6).
C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group; mRNA, messenger RNA; LDH, lactate dehydrogenase; PDHX, pyruvate dehydrogenase complex; PK, pyruvate kinase; SDH, succinate dehydrogenase; UCP, uncoupling protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[a]$p < 0.05$, [b]$p < 0.01$ presented a significant difference.

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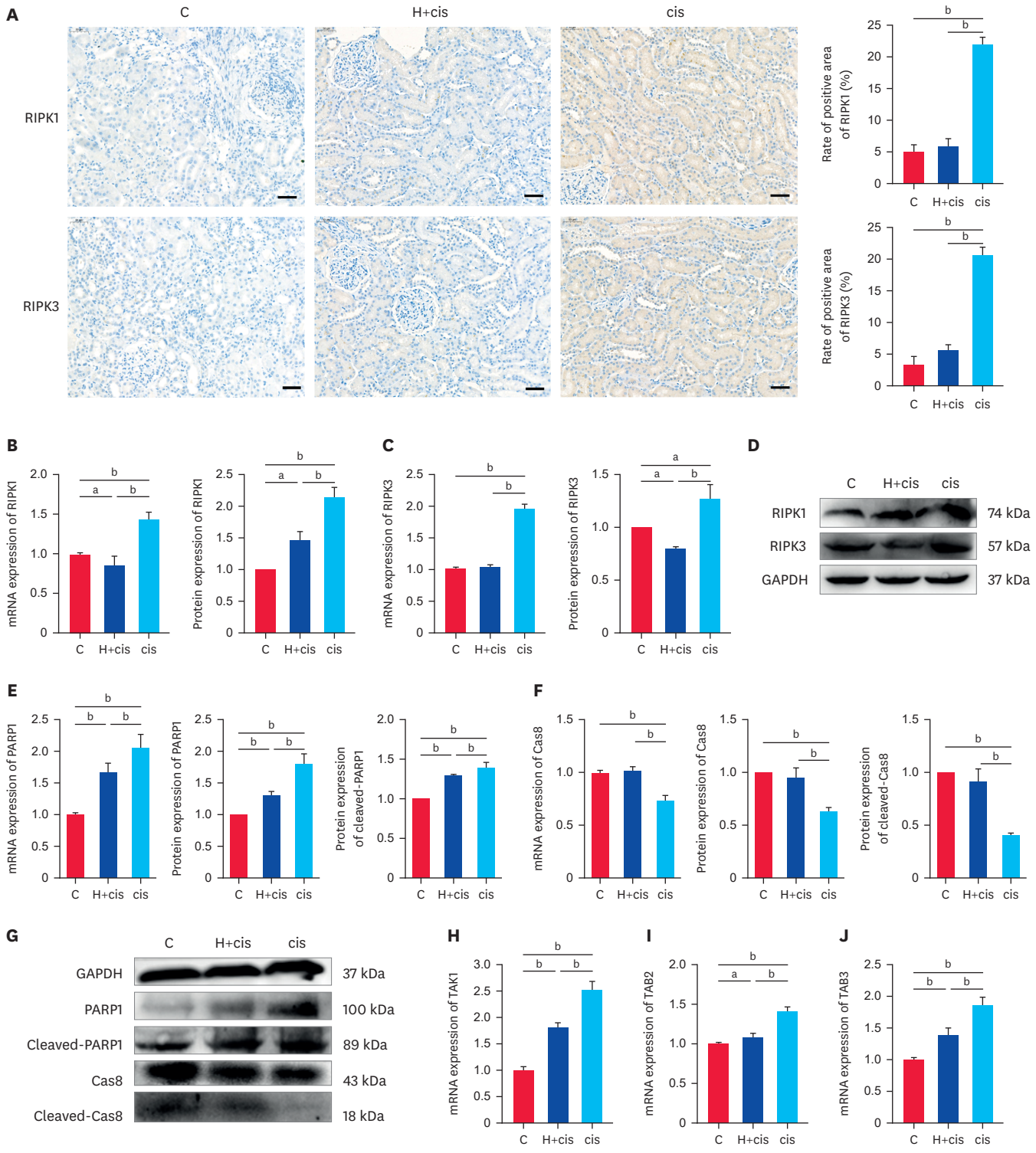

Fig. 5. The expression levels of necroptosis-related genes in canine kidney tissues. (A) Representative images and quantification for immunohistochemistry of RIPK1 and RIPK3. (B-F) The mRNA and protein expression of RIPK1, RIPK3, PARP1 and Cas8. (G-I) The mRNA expression of TAK1, TAB2 and TAB3. Scale bars = 50 μm. GAPDH was selected as the reference of mRNA and protein expressions. Data are represented as the mean ± SD (n = 6).
C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group; RIPK, receptor interacting protein kinase; mRNA, messenger RNA; PARP, poly ADP-ribose polymerase; Cas8, caspase-8; TAK, transforming growth factor-β activated kinase; TAB, TAK1-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[a]$p < 0.05$, [b]$p < 0.01$ presented a significant difference.

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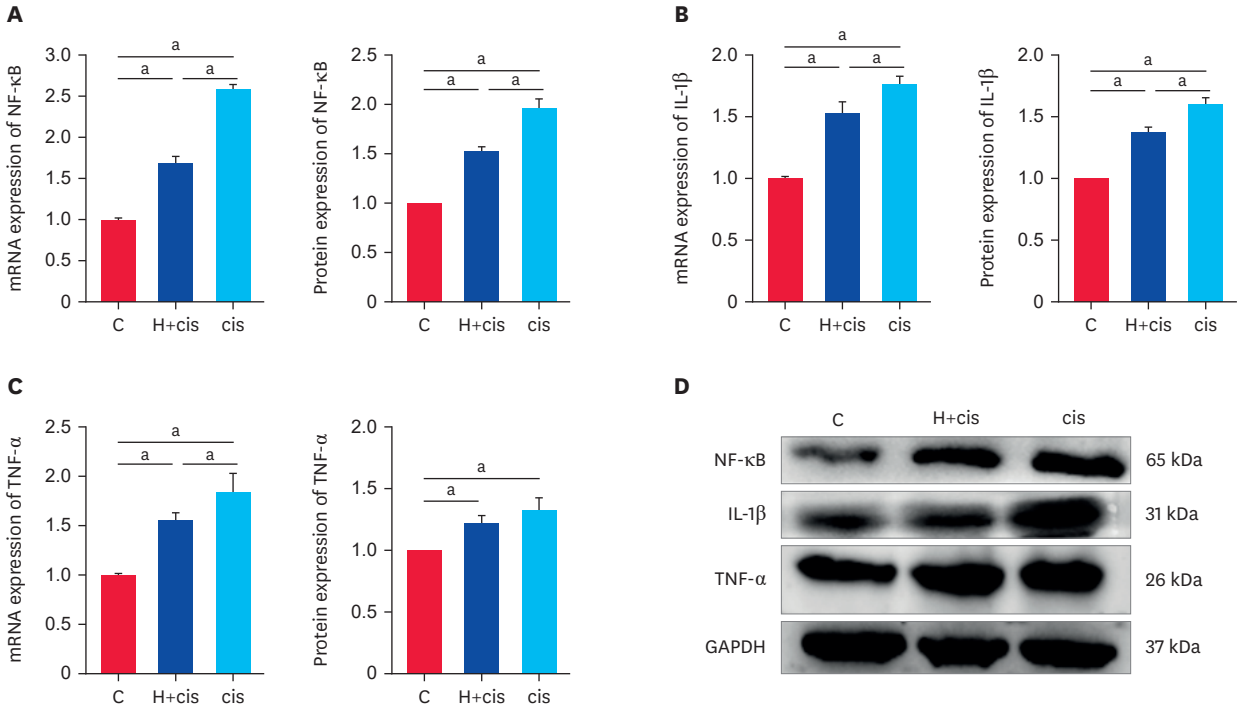

Fig. 6. The expression levels of inflammation-related genes in canine kidney tissues. (A) The related expression of NF-κB. (B) The related expression of IL-1β. (C) The related expression of TNF-α. (D) Western blotting for NF-κB, IL-1β and TNF-α. GAPDH was selected as the reference of mRNA and protein expressions. Data are represented as the mean ± SD (n = 6).
C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group; NF-κB, nuclear factor kappa B; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; mRNA, messenger RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[a]$p < 0.01$ presented a significant difference.

Moreover, the mRNA and protein expression of PARP1 in the cis group was significantly higher than that in the C and H+cis groups ($p < 0.01$; **Fig. 5E and G**). Compared with the C and H+cis groups, the mRNA and protein expressions of Cas8 in the cis group were significantly decreased ($p < 0.01$; **Fig. 5F and G**). Furthermore, the mRNA expressions of transforming growth factor-β activated kinase (TAK) 1, TAK1-binding protein (TAB) 2, and TAB3 in the cis group were enhanced significantly compared with those in the C group ($p < 0.01$), whereas after H_2S treatment, their mRNA expression levels decreased significantly ($p < 0.01$; **Fig. 5H-J**).

Level of inflammatory response in canine kidney tissues

Compared with the C group, the mRNA and protein expressions of NF-κB, IL-1β, and TNF-α in the cis group increased significantly ($p < 0.01$; **Fig. 6**). Among them, NF-κB mRNA expression increased the most in the cis group, which was approximately 2.5 times higher than that in the C group (**Fig. 6A**). After H_2S pretreatment, the mRNA and protein expressions of NF-κB, IL-1β, and TNF-α decreased. As expected, compared with the cis group, the protein expression of TNF-α in the H+cis group decreased the least, and no significant difference was observed between the two groups ($p > 0.05$; **Fig. 6C**).

DISCUSSION

AKI is the acute decline of renal function in a short time due to various reasons. Dialysis and kidney transplantation are effective AKI treatments, but they are difficult to apply in

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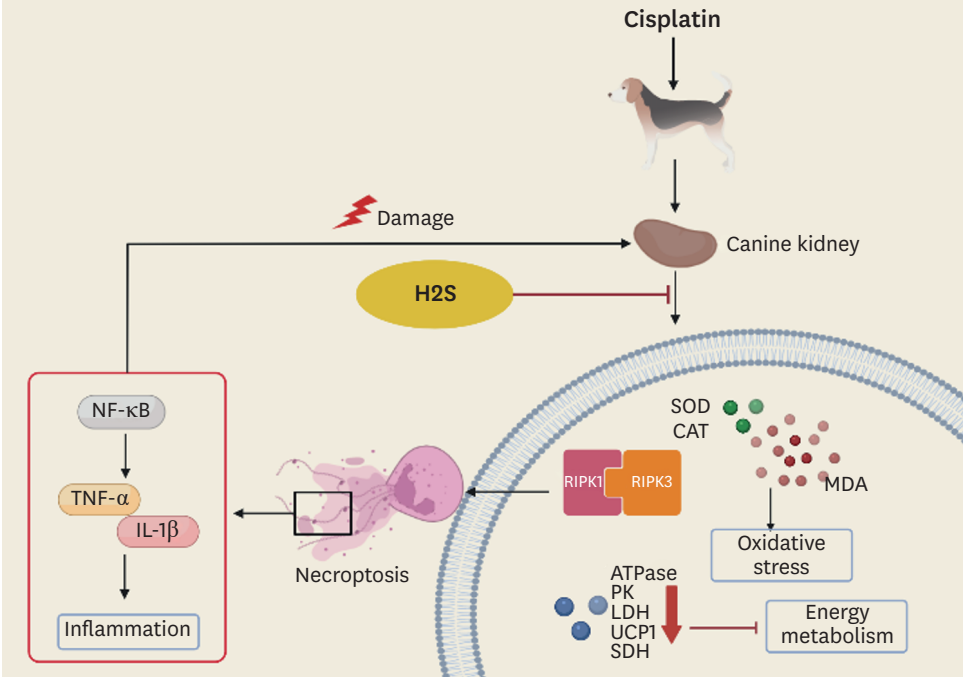

Fig. 7. The schematic diagram of protective effect of H_2S on canine AKI induced by cisplatin. Cisplatin induces necroptosis of canine renal tissues and triggers inflammation, as well as reduces renal antioxidant capacity and energy metabolism. These are notably improved by H_2S.
H_2S, hydrogen sulfide; AKI, acute kidney injury; NF-κB, nuclear factor kappa B; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; SOD, superoxide; CAT, catalase; RIPK, receptor interacting protein kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; UCP, uncoupling protein; SDH, succinate dehydrogenase; MDA, malondialdehyde.

veterinary clinics because of their high cost. Therefore, effective methods for AKI prevention are of great significance in the field of veterinary medicine. In the present study, we demonstrated that H_2S protected against cisplatin-induced canine kidney injury through the inhibition of necroptosis, inflammation, and oxidative stress. Moreover, our study confirmed that cisplatin reduces energy metabolism in canine kidney tissues, whereas H_2S improves this situation (**Fig. 7**).

Necroptosis is a type of programmed necrosis which is of central pathophysiological relevance in various diseases such as myocardial infarction [22], atherosclerosis [23], and ischemia-reperfusion injury [24]. TNFR regulation is the classic pathway of necroptosis, during which two complexes are formed. Complex I is mainly composed of TRADD, RIPK1, TRAF2, and TRAF5. If RIPK1 is ubiquitinated, it binds to TAK1, TAB2, and TAB3 and further activates NF-κB to inhibit cell death [25]. Conversely, if RIPK1 is deubiquitinated, it forms complex II with RIPK3, TRADD, and Cas8, which can initiate necroptosis under conditions of Cas8 inactivation [26]. Studies have shown that necroptosis is involved in various pathological conditions of the kidney. Newton et al. [27] demonstrated that RIPK3 deficiency can improve kidney ischemia-reperfusion injury in mice. Xu et al. [28] revealed that knocking out mice necroptosis key genes RIPK1 and RIPK3 can attenuate the damage caused by cisplatin to the kidney, which indicates that necroptosis is one of the main mechanisms of cisplatin-induced AKI. Therefore, inhibiting the expression of key necrosis factors (such as RIPK1 and RIPK3) may alleviate cisplatin nephrotoxicity. In this regard, we evaluated the renoprotective effect of H_2S and found that it can weaken the expression of RIPK1, RIPK3, and PARP1, and simultaneously, it can enhance Cas8 activity. Thus, H_2S can relieve cisplatin-induced necroptosis of the canine kidney.

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AKI often manifests as an inflammation of the kidney tissue [29]. TNF-α is a pro-inflammatory factor mainly produced by macrophages, which can induce substances such as IL and interferon to cause inflammation [30]. Moreover, TNF-α can activate the NF-κB pathway, thereby inducing the production and release of pro-inflammatory factors IL-1 and IL-6. Gong et al. [31] demonstrated that the protein expressions of TNF-α and IL-6 in rat kidneys were significantly upregulated on the third day after cisplatin injection into rats. Furthermore, studies have demonstrated that necroptosis plays a crucial role in inflammation and is involved in multiple inflammatory diseases. Vince et al. [32] illustrated that the activation of RIPK3 can generate bioactive IL-1β, which is a potent inflammatory cytokine. Welz et al. [8] suggested that the inhibition of RIPK3-induced necrosis can prevent the inflammation of intestinal epithelial cells in mice. Moreover, several studies have indicated that necroptosis induced by RIPK3 promotes the production of some cytokines and inflammatory factors, thereby inducing inflammation [33,34]. Studies have demonstrated that RIPK1 triggered a second wave of cell death in AKI, whereas RIPK1 potentially regulated inflammation in a way unrelated to cell death [35,36]. In the present study, optical microscopy observation revealed that H_2S alleviates the pathological damage to the canine kidney caused by cisplatin. Further detection at the molecular level showed that H_2S reduced the expression of pro-inflammatory factors (including IL-1β, NF-κB, and TNF-α).

Studies have indicated that cisplatin can cause renal oxidative stress and induce damage kidney; in detail, the content of MDA increased, and the activity of glutathione (GSH) decreased [37]. Furthermore, Waly et al. [38] showed that cisplatin induced oxidative stress in human kidney (HEK 293) cells through the reduction of the activities of SOD, GSH, and CAT. Additionally, Zhang et al. [39] demonstrated that RIPK3 mediates oxidative stress, which can induce necroptotic cell death and inflammation. In this setting, extenuating oxidative stress-induced necroptosis through H_2S seems to be effective against renal inflammation. In this study, we found that H_2S restored the activity of antioxidant enzymes (including SOD and CAT) and decreased the total content of MDA, which suggested that H_2S could increase antioxidant capacity in cisplatin-induced canine AKI.

Additionally, many other crucial factors transmit and execute necrotic signals. A recent study indicated that glycolytic pyruvate played a novel anti-necroptotic role in ischemic stress of mice gut [40]. Another report revealed that ATPase activities were inhibited, and several energy metabolism–related gene expressions decreased during necroptosis [41], which suggested that energy metabolism is related to necroptosis. Furthermore, Yang et al. [42] observed an imbalance of energy metabolism in canine kidney tissues in a lipopolysaccharide-induced canine septic AKI. Here, we detected the expression of energy metabolism–related genes (including PK, SDH, UCP1, PDHX, and LDH) and the activities of Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, and Ca^{2+}-ATPase. Our results showed that cisplatin reduced the level of canine kidney energy metabolism, and H_2S can mitigate this condition.

In summary, we successfully established a cisplatin-induced kidney injury model in dogs and demonstrated that H_2S has a powerful protective effect on cisplatin-induced AKI through the enhancement of the antioxidant capacity and energy metabolism level, as well as the reduction of cell necroptosis and inflammation. These findings provide new and valuable clues for the treatment of canine AKI and are of great significance for AKI prevention in veterinary clinics. Simultaneously, our study enriched the understanding of the H_2S effect on necroptosis and inflammation, which may provide new insights into the physiological role of H_2S.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Scr and Bun levels in dogs. In the pre-test, 12 adult healthy beagles were randomly divided into four groups: C group, H group, H+cis group and cis group. Blood samples were taken 72 h after cisplatin injection to test the levels of Scr and Bun. Results showed that there was no significant difference in Scr and Bun between H group and corresponding C group, indicating that the injection of NaHS solution had no effect on canine kidney. Therefore, in order to respect and protect the experimental animals, the hydrogen sulfide group was not set in our formal experiment.

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