Hydrogen sulfide protects from cisplatin-induced acute kidney injury via attenuating inflammation activated by necroptosis in canine

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Research

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Abstract

Background Cisplatin, as an effective anti-cancer drug, has significant effects on a variety of solid tumors such as ovarian cancer and malignant lymphoma. However, cisplatin has strong nephrotoxicity, which greatly limits its clinical application. Unfortunately, the way to validly inhibit cisplatin-induced kidney damage remain poor understood currently. Hydrogen sulfide (H$_2$S) was considered to be the third new gas signal molecules, involved in various physiological functions of the body. This present study was designed to investigate the effect of H$_2$S on cisplatin-induced acute kidney injury (AKI) and the involved mechanisms in canine.

Results Cisplatin-inject canine developed severe AKI symptom as indicated by renal dysfunction and pathological changes. Whereas H$_2$S attenuated the serum creatinine (Scr), blood urea nitrogen (Bun) level and renal tubular damage. Cisplatin induced necroptosis and regulated the corresponding protein expression of RIPK1, RIPK3, PARP1, and then activated inflammatory factors expression such as TNF-α, NF-κB, IL-1β in canine kidney tissues. Cisplatin also triggered oxidative stress and affected energy metabolism. However, H$_2$S significantly improved necroptosis and inflammation, manifested by increasing Cas8 activity and the expression of anti-inflammatory factors such as IL-4, IL-10 and IFN-γ. At the same time, the antioxidant capacity and energy metabolism levels of canine kidney were notably improved.

Conclusion Collectively, our results suggest that H$_2$S protect kidney from cisplatin-induced AKI via mitigating necroptosis and inflammation. Besides, H$_2$S improved the level of energy metabolism and displayed a potency in cisplatin-induced oxidative stress. These provided a new idea for reducing cisplatin nephrotoxicity.

Highlights

- The level of energy metabolism was weakened due to cisplatin in canine kidney tissue.
- Hydrogen sulfide enhances antioxidant capacity in cisplatin-induced acute kidney injury.
- Hydrogen sulfide protect canine kidney tissue from cisplatin via decreasing inflammation mediated by necroptosis.

1 Introduction

Acute kidney injury (AKI), as an independent risk factor for mortality, is a clinical syndrome characterized by rapid decline of renal function. As one of the most common complications in hospitalized patients, AKI increases the risk of death by 10 to 15 times and result in a mortality rate of 50%, which it accounts for approximately 2 million deaths per year worldwide [1, 2]. There have a large-scale multicenter epidemiological survey of critically ill patients showed that AKI induced by drug nephrotoxicity accounted for 19% [3]. Cisplatin (cis-diamminedichloroplatinum II, CDDP), as a well-known chemotherapy drug, has a significant therapeutic effect on various solid tumors. However, the use of cisplatin is frequently limited
by various significant side effects especially nephrotoxicity. As well as tubular cell injury and death, cisplatin can cause inflammation of kidney tissue [4, 5]. It has been reported that 30%-40% of cisplatin-treated patients developed AKI [6]. Unfortunately, in the prevention of cisplatin nephrotoxicity, some people have tried to supplement magnesium, and some have tested antioxidants and cisplatin transport blockers in animal experiments, but the results are not satisfactory [7, 8].

Necroptosis is one of the cell death modes of AKI, which is a type of programmed cell death mediated by receptor interacting protein kinase (RIPK) signaling [9]. RIPK1 is a key factor in the initiation of necroptosis, it combines with tumor necrosis factor receptor 1 (TNFR1), TNFR1-associated death domain protein (TRADD) and TNFR-associated factor 2 (TRAF-2) via death domain to form complex I that can induce necroptosis via forming RIPK1/RIPK3/MLKL necrosome in the absence of caspase-8 (Cas8) [10, 11]. In recent years, more and more studies have provided that necroptosis is associated with inflammation. For instance, Welz et al. have found that RIPK3 gene deficiency prevented the development of inflammation and cell death in both the small intestine and colon of mice [12]. Murakami et al. have identified that programmed necrosis promoted inflammation by regulating the release of intracellular damage-associated molecular pattern in mice with retinal degeneration [13]. Additionally, RIPK3 can activate glutamate-ammonia ligase, thereby increasing the decomposition of glutamate, and further mitochondrial glutamate catabolism leads to local free ammonia accumulation and increases ROS expression [13, 14]. This suggest that necroptosis may be associated with oxidative stress.

Hydrogen sulfide (H$_2$S) was considered to be the third endogenous signaling gaseous transmitter along with nitric oxide (NO) and carbon monoxide (CO), which plays an important role in various tissues in both health and disease [15]. In fact, H$_2$S was initially identified as a harmful exogenous gas with pungent smell, which can cause damage to kinds of tissues and organs of the body [16]. Until 1996, Abe and Kimura discovered that H$_2$S can be produced by a series of enzymatic reactions in mammals [17], the physiological function of H$_2$S has been gradually recognized. In general, H$_2$S is synthesized from L-cysteine via three enzymes: cystathionine-β-synthase (CBS), cystathionine-β-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) [18]. 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$_2$S played an important role in inflammation. For example, H$_2$S can induce apoptosis of neutrophils to reduce inflammation [19]. Administration of H$_2$S to rats with colitis can down-regulate the expression of pro-inflammatory cytokine tumor necrosis factor α (TNF-α), whereas inhibiting the synthesis of H$_2$S in healthy rats induced inflammation in the small intestine and colon [20]. Furthermore, Chen et al. have showed that exogenous administration of NaHS could alleviate airway inflammation [21]. In addition, some recent reports have highlighted that H$_2$S displayed significant antioxidant properties, which could up-regulate the expression of key antioxidant enzymes and remove ROS [22, 23]. King et al. have reported that the level of oxidative stress in mice with myocardial infarction was increased after CSE knockout, and the myocardial injury was aggravated, both of which were alleviated via exogenous H$_2$S [24].
Improving the pathogenesis of cisplatin-induced AKI is of great significance to the clinical application of cisplatin drugs. In our present study, we built a canine AKI model with cisplatin, and examined whether H₂S attenuates cisplatin nephrotoxicity, and explored how H₂S protects kidney from cisplatin nephrotoxicity. To our knowledge, previous studies of cisplatin-induced AKI have focused on mice, there are fewer reports about AKI in dogs and the effect of H₂S on AKI. This study revealed a possible mechanism of H₂S alleviating cisplatin-induced AKI in dogs, which will provide more possibilities for clinically reducing the side effects of cisplatin.

2. Materials And Methods

2.1 Preparation of animals

All procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural. Twenty-four adult male beagles (8–12 kg) were divided randomly into three groups (n = 6 per group, the remaining two dogs in each group were standby for any unexpected condition). The laboratory staff cleaned the kennel regularly to ensure the environment is good; the kennel temperature is controlled at 18–26°C. All dogs were given free access to standard food and water during the study. Dogs in the cis group were injected with 5 mg /kg body weight cisplatin, and control dogs received an injection of equal volume of saline. As for H + cis group, dogs were injected with 50 mM NaHS solution (1 mg / kg / h) 30 min before cisplatin injection (5 mg / kg) and then inject once daily. Dogs were anesthetized 72 h after the cisplatin injection. The left kidney tissues and blood were quickly collected, blood sample was collected for blood urea nitrogen (Bun) and serum creatinine (Scr) measurement, the upper half of left kidney was quickly removed and fixed in 10% phosphate-buffered formalin for hematoxylin-eosin staining, and the lower half of left kidney was quickly removed and frozen in liquid nitrogen and then stored at −80 °C.

2.2 Serum analysis

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

2.3 Histopathological examination

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

2.4 Detection of antioxidant levels

The kidney tissues were homogenized (1:10 w/v) with a glass Teflon homogenizer (Heidolph SO1 10R2RO) in physiological saline. The homogenate was centrifuged at 700 × g for 30 min at 4 °C to obtain the supernatant to measure the activities of SOD, GSH, CAT as well as MDA, H₂O₂ and NO content levels.
by the detection kits (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer protocols.

2.5 Detection of ATPase

The activities of Na\(^+\)-K\(^+\)-ATPase, Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase, and Ca\(^{2+}\)-ATPase were determined using the appropriate assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol using 10% tissue homogenates. The activities of Na\(^+\)-K\(^+\)-ATPase, Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase were measured by quantifying the inorganic phosphorus (Pi) production from the conversion of ATP to ADP at 660 nm using the molybdenum blue spectrophotometric method and were expressed as U/mg.prot. When one type of ATPase was tested, the inhibitors of other types of ATPase were added to depress the hydrolysis of phosphate radicals.

2.6 Quantitative real-time PCR analysis

Total RNA from canine kidney tissues was extracted using 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). Quantitative real-time PCR was performed on a Light Cycler® 480 System (Roche, Basel, Switzerland) after reverse transcription by using the fast qPCR kit (RR047A, Takara). All of the primers (Table. 1) were designed by Premier Software (PREMIER Biosoft International, USA) for q-PCR. The relative mRNA level was calculated according to the method of \(2^{-\Delta\Delta C_t}\), accounting for gene-specific efficiencies was normalized to the mean mRNA expressions of GAPDH.

2.7 Immunofluorescence staining

The kidney sections was treated with 0.01 M sodium citrate buffer (PH 6.0) by a microwave-based antigen retrieval technique for 20 min at 95°C was used followed by 10 min 3% H\(_2\)O\(_2\) to block endogenous peroxidase activity, incubated with RIPK1 (1:500; Bioss, China), RIPK3 (1:500; Bioss, China) antibodies for 24 h at 4°C and secondary antibodies for 30 min at 37°C. After staining with DAB the slides were visualized with microscope.

2.8 Western Blot analysis

The protein samples were separated by 8%, 10% and 12% SDS-PAGE and were transferred to PVDF membranes (Merck Millipore, USA, Cat# ISEQ. 00010, LOT# R6PA4145H). 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: PK (1:1000; Wanlei, China), UCP1 (1:1500; Wanlei, China), SDH (1:500; Bioss, China), PDHX (1:500; Afflnlt, China), LDH (1:1000; Wanlei, China), PARP1 (1:500; Proteintech, USA), Cas8 (1:1000; CST, USA), NF-κB (1:500; Wanlei, China), IL-1β (1:1000; Wanlei, China), IL-6 (1:1000; Wanlei, China), TNF-α (1:500; Wanlei, China), COX2 (1:1000; Wanlei, China), iNOS (1:500; Wanlei, China), Hsp60 (1:1000; Wanlei, China), Hsp70 (1:500; Wanlei, China), IL-4 (1:500; Wanlei, China), IL-10 (1:500; Wanlei, China), IFN-γ (1:500; Wanlei, China). After washing three times for 15 min each with PBST, the membranes were incubated for 2 h at 37 °C with peroxidase-conjugated secondary antibodies against rabbit IgG (Santa
Cruz Biotechnology, Argentina, Cat# sc-2357, RRID: AB_628497). After washing three times for 15 min each, the bound antibodies were visualized by chemiluminescence using the ECL-plus reagent (GE Healthcare, Buckinghamshire, UK). The GAPDH content was analyzed as the loading control using a rabbit polyclonal antibody.

2.9 Statistical analysis

Statistical analyses of all data were performed using GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA). The significant values \( (P < 0.05) \) were obtained by One-way ANOVA. 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 \( P < 0.05 \).

3. Result

3.1 \( \text{H}_2\text{S} \) attenuated cisplatin-induced renal injury in canine

We tested six samples in each group and found that the content of Bun and Scr increased significantly after cisplatin treatment \( (P < 0.01) \), while \( \text{H}_2\text{S} \) could improve the change (Fig. 1A). In addition, we observed canine kidney tissues stained by hematoxylin and eosin (H&E) in the C group, H + cis group and cis group. The histopathological changes in renal tissues are shown in Fig. 1B. The kidney tissues in the corresponding C groups displayed normal morphologies, including the glomeruli and tubulesballoon are normal, the saccule cavity is obvious, and the vessel wall is clear. However, some features of renal pathological damage appeared in the cis group, after cisplatin administration, the canine kidney tissue showed glomerular fibrosis (black arrow), tubular atrophy (red arrow), as well as renal interstitial hyperplasia (green arrow). Besides, we also found the infiltration of inflammatory cells in renal tissue (yellow arrow). Obviously, in the hydrogen sulfide group, renal pathological damage was relieved, but there were still some changes compared with the normal group, including renal balloon occlusion (white arrow) and fibrinoid necrosis of vascular wall (blue arrow).

3.2 The antioxidant capacity in canine kidney tissues

The results of antioxidant activity of canine kidney tissues were as follows. Compared with the corresponding C groups, the activitis of SOD, GSH and CAT in cis groups were significant decreased \( (P < 0.05 \) or \( P < 0.01) \), after the addition of \( \text{H}_2\text{S} \), the above antioxidant enzyme activities were restored (Fig. 2A, B and C). In addition, there were no significant difference between the C group and H + cis group about the contents of MDA, \( \text{H}_2\text{O}_2 \) and NO \( (P > 0.05) \), whereas, the above substances in cis group enhanced compared to the corresponding C group and H + cis group \( (P < 0.01) \) (Fig. 2D, E and F).

3.3 ATPase activities and energy metabolism-related expressions in canine kidney tissues
ATPase activity results appear in Fig. 3A-C. The activity of all ATPase were weakened after cisplatin treated. Notably, the most decreased was Ca\textsuperscript{2+}-ATPase activity in cis group, which decreased by about 28% (Fig. 3C). Besides, there was also significant difference in Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase activity under cisplatin (P<0.01) (Fig. 3B). Whereas in H + cis group, all ATPase activity recovered. At the same time, the expression of some energy metabolism genes was also detected. As shown in Fig. 3D-I, the expression levels of pyruvate kinase (PK), uncoupling protein 1 (UCP1), succinate dehydrogenase (SDH), pyruvate dehydrogenase complex (PDHX) and lactate dehydrogenase (LDH) were markedly decreased (P<0.01) after cisplatin treatment. Moreover, the expression of all energy metabolism related genes in H + cis group were elevated significantly compared to that cis group (P<0.01). Among them, the relative expression of PK and the protein expression of LDH in the H + cis group was slightly higher than that C group.

### 3.4 The 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\textsubscript{2}S in canine kidney tissues are shown in Fig. 4. Cisplatin treatment significantly increased the mRNA and protein levels of necrosis genes including RIPK1, RIPK3 and PARP1 (P<0.01) (Fig. 4A-C), while Cas8, as genes that induce apoptosis and inhibit necrosis, were decreased significantly (P<0.01) (Fig. 4D). Among them, the most increased was PARP1 mRNA expression in cis group, which about doubled compared to the corresponding C group (Fig. 4C). However, after H\textsubscript{2}S treatment, the levels of all necrosis genes decreased, at the same time, the levels of Cas8 enhanced. Obviously, the protein expression levels of RIPK1 and PARP1 in H + cis group decreased slightly compared to that cis group (P>0.05) (Fig. 4A, C), only decreased by about 5% and 3% respectively. Furthermore, the mRNA expression of TAK1, TAB2 and TAB3 in cis group were enhanced significantly compared to that C group (P<0.01), whereas H\textsubscript{2}S treatment, their mRNA expression levels decreased significantly (P<0.01) (Fig. 4E-G).

### 3.5 The level of inflammatory response in canine kidney tissues

As shown in Fig. 5, there were significant difference in the mRNA expression level of pro-inflammatory genes (including IL-1\textbeta, IL-6, TNF-\textalpha, NF-\kappaB, COX2 and iNOS) after cisplatin treatment (P<0.01). Among them, the most increased was the COX2 mRNA expression level in cis group, which was about 5.5 times of the corresponding C group (Fig. 5E). Besides, the pro-inflammation genes mRNA expression levels in the H + cis group were down-regulated obvious compared to that cis group (P<0.01). Furthermore, the mRNA expression levels of anti-inflammatory genes (including Hsp60, Hsp70, IL-10, IL-4 and IFN-\gamma) in cis group were decreased significantly compared to that corresponding C group (P<0.01), while H\textsubscript{2}S could improve this change (Fig. 5G-K). Moreover, the protein expression level trend of inflammation related genes were consistent with that of mRNA expression level.
4. Discussion

Cisplatin is a powerful anticancer drug, its hydrated or hydroxylated metabolite are mainly excreted through the kidney. Due to nephrotoxicity, the clinical application of cisplatin has been limited. Unfortunately, there is currently no effective way to prevent the kidney damage caused by cisplatin. In the present study, we demonstrated that H₂S protected against cisplatin-induced canine kidney injury by limiting necroptosis, inflammation and oxidative stress. Moreover, our study confirmed that cisplatin reduces the energy metabolism of the kidney tissues, whereas H₂S can improve this situation.

Necroptosis is a type of programmed necrosis, which is of central pathophysiological relevance in a variety of disease states such as myocardial infarction [25], atherosclerosis [26] and ischemia-reperfusion injury [27]. TNFR regulation is the classic pathway of necroptosis, during this process two complexes are formed. Complex I is mainly composed of TRADD, RIPK1, TRAF2 and TRAF5, if RIPK1 is ubiquitinated, it will bind to transforming growth factor-β activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), TAB3 and further activate nuclear factor kappa-B (NF-κB) to inhibit cell death [28]. On the other hand, if RIPK1 is deubiquitinated, it will form complex II with RIPK3, TRADD, Cas8 that can initiate necroptosis under conditions of inactivation of Cas8 [29]. Previous studies have shown that necroptosis is involved in various pathological conditions of the kidney. Newton et al. have shown that RIPK3 deficiency can improve kidney ischemia-reperfusion injury in mice [30]. Xu et al. have found that knocking out mice necroptosis key genes RIPK1 and RIPK3 can attenuate the damage caused by cisplatin to the kidney [31], which indicates that necroptosis is one of the main mechanism of cisplatin-induced AKI. Therefore, inhibiting the expression of key necrosis factors (such as RIPK1 and RIPK3) may be a way to alleviate cisplatin nephrotoxicity. In this regard, we evaluated the renoprotective effect of H₂S and found it can weaken the expression of RIPK1, RIPK3 and PARP1, at the same time enhanced Cas8 activity. These suggest that H₂S relieve cisplatin-induce necroptosis of canine kidney.

Furthermore, there are reports have demonstrated that necroptosis plays an important role in inflammation and is involved in multiple inflammatory diseases. Vince et al. have illustrated that activation of RIPK3 can generate bioactive interleukin-1β (IL-1β) that is a potent inflammatory cytokine [32]. The research of Welz et al. suggested that inhibiting RIPK3-induced necrosis can prevent the inflammation of intestinal epithelial cells in mice [12]. Moreover, there are several studies have indicated that the necroptosis induced by RIPK3 promotes the production of some cytokines and inflammatory factors, thereby inducing inflammation [33, 34]. There are other reports have shown that RIPK1 triggered a second wave of cell death in AKI, while RIPK1 may regulate inflammation in a way unrelated to cell death [35, 36]. In the present study, we observed through optical microscopy that H₂S alleviate the pathological damage of canine kidney caused by cisplatin, such as glomerular fibrosis, interstitial hyperplasia and renal tubular atrophy, etc. Further detection at the molecular level shown that H₂S reduced the expression of pro-inflammatory factors (including IL-1β, IL-6, TNF-α, NF-κB, COX2, iNOS) and increased several anti-inflammatory factors (including IL-4, IL-10, IFN-γ) activities, as well as heat shock protein 60 (Hsp60) and Hsp70. Hsp as a highly conserved cellular stress proteins synthesized in organism, are related to
necroptosis and inflammation. There are studies have revealed that Hsp70 could suppress RIPK1-dependent necroptosis [37]. Zonneveld et al. have reported that a recently epitopes derived from Hsp60 can ease inflammation via reducing TNF-α and increasing IFN-γ [38], which are consistent with our results.

Previous studies have indicated that cisplatin could cause renal oxidative stress and induce damage kidney, in more details, the change was that the content of malondialdehyde (MDA) increased and the activity of glutathione (GSH) decreased [39]. Besides, Waly et al. have shown that cisplatin induced oxidative stress in human kidney (HEK 293) cells via reducing the activities of superoxide (SOD), GSH and catalase (CAT) [40]. Additionally, Zhang et al. have demonstrated that RIPK3 mediates oxidative stress could induce necroptotic cell death and inflammation [41]. In this setting, extenuating oxidative stress-induced necroptosis via H$_2$S seem to be an effective way to against renal inflammation. In the current study, we found that H$_2$S restored the activity of antioxidant enzymes (including SOD, GSH and CAT) and decreased the total content of MDA, H$_2$O$_2$ and NO, which suggested that H$_2$S could raise antioxidant capacity in cisplatin-induced canine AKI.

In addition to the above, there are many other important factors that transmit and execute necrotic signals. A recent study have indicated that glycolytic pyruvate played a novel anti-necroptotic role in ischaemic stress of mice gut [42]. Another report have shown that the activities of ATPase were inhibited and several energy metabolism related genes expressions decreased during necroptosis [43], which suggested energy metabolism seems to be related to necroptosis. 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 shown that cisplatin reduced the level of canine kidney energy metabolism, and H$_2$S could improve this situation.

5. Conclusion

In summary, here we have demonstrated that H$_2$S had a powerful protective effect on cisplatin-induced AKI via enhancing the antioxidant capacity and the level of energy metabolism, as well as improving cell necroptosis and inflammation (Fig. 6). These finding provide new and valuable clues for the treatment of cisplatin nephrotoxicity and AKI. Simultaneously, our study enrich the understanding of the H$_2$S effect on necroptosis and inflammation, which may provide new insight into the physiological role of H$_2$S.

Declarations

Acknowledgment

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Authors’ contribution
SW and EX designed the experiments, SW, RG, HJ, YF, MH, SJ and TG performed the experiments. SW and XR analyzed the experimental data. SW and YL wrote this paper. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are available from the corresponding author by request.

**Ethics approval and consent to participate**

Not applicable.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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Table

| Table. 1 mRNAs Primer Sequences |
| Gene   | Forward 5' to 3'                          | Reverse 5' to 3'                          |
|--------|------------------------------------------|------------------------------------------|
| PK     | GACCGGCCGCGCTACCTGAG                     | GCTGCTGCTGCTGGAAGAAGG                   |
| UCP1   | AGAGGCGAGTGGCGTCTCGC                    | GCTGCTCCTCCCGCTATTACAC                  |
| SDH    | GCGGAGCTCTCTGGAGCTGAG                   | GGCTGGTCCTGGAGAAATGCTG                  |
| PDHX   | CCTGGAGGGCGGGCTGCGAC                    | GCGCTCGGTGGCTGTAAG                      |
| LDH    | CGCTGCTGGCTACAACCTCAC                   | GCTGCTGGACACTGGGAAAC                    |
| RIPK1  | CCGATTCTCTGCGGAACCTGT                   | ATCCACATGTGTCGCGTTCC                   |
| RIPK3  | GCCCTTCCTGTAACCAGATG                    | ACACGACGGCGCAACTGAC                    |
| PARP1  | GGCTCTGATGACAGCAGCAGCGAAGC             | TTTCTGAGCTGCTTGCCGCTTCCCTC             |
| Cas8   | TGGCTCTGATGGGAGCGAGGACG                 | GGGCTTGGCCTGCAAGGAGAG                  |
| NF-κB  | TGAACATGACAGCAGGAAAG                   | AGCAAGCTGATGGACCTGT                    |
| TAK1   | GCCATCATCCGCAACCTCATCC                 | AGCAGTCCACAGCGCCCTCATCC                |
| TAB2   | GGAAGCAGGACTCTAAGCGACAGG               | GCGTTGGAGGAATCTGGAGCTGGT               |
| TAB3   | GTTGTGGCTGCTACTCCGGAACCTACT            | ATGGTTGTTGAGGCTGCTGTGAAG               |
| IL-1β  | GGCAGTCACACAGCGATCAACA                  | GCAATCCATGAGATCCAGTT                   |
| IL-6   | TGGGAAGGCTGAGAGAGAGA                   | GGTGTTCCCTCAAGTGTGGT                   |
| TNF-α  | GCTAGGCGACCAGGTTATGGA                  | GCTGAGCATCCGTTGGGT                    |
| COX2   | AGGCAAGCAGAGACTGGGA                    | CTGACTTACACAAGAACACC                   |
| iNOS   | CCGAAGGAGCAAGCAG                       | AGGTTGAGACTGGGAGTAG                    |
| Hsp60  | CCCTGGCTCTCATCTCATCTGG                  | ACGGCTACGCGACAGCTGTA                   |
| Hsp70  | GAACCTGGACAGAGGCTGGT                   | CTCACTAGGTGGT                        |
| IL-4   | CTGAAGGCTCTGGTTATA                     | CTCGATCTTGCGTCCCTC                    |
| IL-10  | CACGACCCAGACATCAAGAACCAC               | GATGCCTTGGCTGTAGACG                    |
| IFN-γ  | ATGCTGCGCTTCTTCCAGTT                   | CTGACTATCCAAAAGAACCACC                 |
| GAPDH  | CCGCTCTATGAGGCTAGC                     | CTGACTGCTGGTGTA                       |

**Figures**
Figure 1

H2S attenuated cisplatin-induced renal injury in canine. (A) shows the results of urea nitrogen and serum creatinine, (B) represents histopathological changes for renal tissue in canines. Data are expressed as the mean ± SD (n = 6). ** P < 0.01 presented a significant difference. C, control group; H+cis, hydrogen sulfide and cisplatin group, cis, cisplatin group; magnification 200×, scale bars = 50 μm. White arrow: renal balloon occlusion; blue arrow: vascular wall fibrinoid necrosis; black arrow: glomerular fibrosis; green arrow: interstitial hyperplasia; yellow arrow: inflammatory cell infiltration; red arrow: renal tubular atrophy.
Figure 2

Antioxidant levels of kidney tissues in canine. The activity of SOD (A), GSH (B), CAT (C) and the total content of MDA (D), H2O2 (E), NO (F) in canine kidney tissues. Data are expressed as the mean ± SD (n = 6). C, control group; H+cis, hydrogen sulfide and cisplatin group, cis, cisplatin group; # P < 0.05, ## P < 0.01 presented a significant difference; * P < 0.05, ** P < 0.01 presented a significant difference compared to the C group.
Figure 3

Detection of canine kidney tissues energy metabolism level. (A) represents the activity of Na+-K+-ATPase; (B) represents the activity of Ca2+-Mg2+-ATPase; (C) represents the activity of Ca2+-ATPase; (D) represents the mRNA and protein expressions of PK; (E) represents the mRNA and protein expressions of Ucp1; (F) represents the mRNA and protein expression of SDH; (G) represents the mRNA and protein expression of PDHX; (H) represents the mRNA and protein expressions of LDH. GAPDH was selected as the reference of mRNA and protein expressions. C, control group; H+ cis, hydrogen sulfide and cisplatin group; cis, cisplatin group. Data are represented as the mean ± SD (n = 6), # P < 0.05, ## P < 0.01 presented a significant difference; * P < 0.05, ** P < 0.01 presented a significant difference compared to the C group.
Figure 4

The expressions of necrosis relative genes in canine kidney tissues. The expression of RIPK1 (A) and RIPK3 (B), PARP1 (C), Cas8 (D) in canine kidney tissues, (E) (F) (G) represent the mRNA expressions of TAK1, TAB2 and TAB3. Scale bars = 50μm. GAPDH was selected as the reference of mRNA and protein expressions. C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group. Data are
represented as the mean ± SD (n = 6), § § P < 0.05, ## P < 0.01 presented a significant difference; * P < 0.05, ** P < 0.01 presented a significant difference compared to the C group.

**Figure 5**

The expressions of inflammation relative genes in canine kidney tissues. The mRNA and protein expressions of IL-1β (A), IL-6 (B), TNF-α (C), NF-κB (D), COX2 (E), iNOS (F), Hsp60 (G), Hsp70(H), IL-4 (I), IL-10 (G), IFN-γ (K) in canine kidney tissues. GAPDH was selected as the reference of mRNA and protein
expressions. C, control group; H+cis, hydrogen sulfide and cisplatin group; cis, cisplatin group. Data are represented as the mean ± SD (n = 6), # P < 0.05, # # P < 0.01 presented a significant difference; * P < 0.05, ** P < 0.01 presented a significant difference compared to the C group.