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S-ketamine alleviates carbon tetrachloride-induced hepatic injury and oxidative stress by targeting the Nrf2/HO-1 signaling pathway

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1 **S-ketamine alleviates carbon tetrachloride-induced hepatic injury and oxidative**
2 **stress by targeting the Nrf2/HO-1 signaling pathway**

3

4 **Short title:** S-KET protects liver from CCl₄ damage.

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Abstract

The aim of the present study was to investigate the protective effect of S-ketamine (S-KET) against carbon tetrachloride (CCl₄)-induced liver damage and oxidative stress, as well as to elucidate the related underlying mechanisms. Blood was collected to measure biochemical parameters (ALT, AST, ALP, TB and γ -GT) and the liver was harvested for histopathological analysis of enzymes related to the antioxidant response (MDA, SOD, GSH, and GSH-PX). Liver cell apoptosis was evaluated using the TUNEL assay. In addition, the expression levels of apoptosis-related proteins and the Nrf2/HO-1 signaling pathway were detected by western blot analysis to explore potential mechanisms. S-KET protected the liver from CCl₄-induced damage. The changes to the liver biochemical parameters (increased ALT, AST, ALP, TB, and γ -GT) and oxidative stress-related indicators (increased MDA; depleted SOD, GSH, and GSH-PX) induced by CCl₄ were inhibited by S-KET. S-KET also inhibited CCl₄-induced cell apoptosis, the changes in expression of related proteins, and blocked CCl₄-induced liver injury and oxidative stress via activation of the Nrf2/HO-1 signaling pathway. S-KET effectively protected the liver by inhibition of CCl₄-induced damage via up-regulation the Nrf2/HO-1 signaling pathway.

Keywords: S-ketamine; liver injury; inflammatory response; oxidative stress; Nrf2/HO-1 signaling pathway

Introduction

39 Liver injury and disease threaten human health and quality of life(Hoek and
40 Pastorino, 2002; Peng et al., 2018). Oxidative stress due to frequent exposure to
41 detrimental environmental factors, such as H₂O₂, toxins, viruses, and heavy metals,
42 among other toxic substances, leads to cell apoptosis, organ injury, and disease.
43 Therefore, it is necessary to elucidate the mechanisms underlying liver injury and
44 disease in order to identify novel targets for the development of more effective
45 therapeutic strategies.

46 Liver injury is often associated with oxidative stress and inflammation, which
47 activate various mechanisms to protect cells from potential injury and death(Hoek and
48 Pastorino, 2002; Cai et al., 2015). Cells and organisms can adapt to harmful
49 conditions via the activation of stress-related cytokines. As previously described,
50 nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor
51 responsible for regulating the antioxidant response(Buendia et al., 2016). Under
52 physiological conditions, Nrf2 is sequestered in the cytoplasm by kelch-like
53 ECH-associated protein 1 (Keap1), while under stressful conditions, it dissociates
54 from Keap1 and binds to musculoaponeurotic fibrosarcoma proteins, which activates
55 antioxidant response elements and increases transcription of Nrf2-regulated genes,
56 including heme oxygenase-1 (HO-1), glutathione S-transferease, and nicotinamide
57 adenine dinucleotide phosphate quinone dehydrogenase 1. Of these, HO-1 is regarded
58 as an antioxidant-inducible cellular defense factor against oxidative injury that
59 inhibits cell death, modulates inflammation, and promotes the antioxidant effects of

60 Nrf2(Loboda et al., 2016; Chen and Maltagliati, 2018; Zhou et al., 2014). Hence, the
61 Nrf2/HO-1 signaling pathway might play a significant role in the amelioration of
62 oxidative stress(Ibrahim et al., 2020).

63 Ketamine (KET) is a chiral compound with R- and S-stereoisomers that exhibits
64 anesthetic, sedative, and analgesic properties, as well as anti-depressant activities.
65 S-KET is an isomer of KET with superior efficacy and controllability, and fewer side
66 effects as compared to a racemic mixture of R-KET and S-KET(Kose et al., 2012;
67 Paul et al., 2009; Trimmel et al., 2018). In recent years, S-KET has been widely used
68 in Europe and the United States for the treatment for mental health
69 disorders(Wilkinson et al., 2018; Bartlett et al., 2020; Grunebaum et al., 2018;
70 Murrough et al., 2015), post-traumatic stress disorder, and related syndromes, while
71 racemic ketamine is still widely used in China(Chang et al., 2019; Aleksandrova et
72 al., 2017; Zanos and Gould, 2018; du Jardin et al., 2016). Interestingly, it was
73 reported that KET played critical roles in the anti-inflammatory(Gundogdu et al.,
74 2016b; Aswoco et al., 2015), gastroprotective(Suliburk et al., 2006), and protection of
75 ischemic spinal cord injury (Yu et al., 2008). In addition, KET was demonstrated to
76 attenuate liver injury through regulating cyclooxygenase-2 (Suliburk et al., 2005) and
77 exert hepatoprotective effect via mediating HO-1 and its end product carbon
78 monoxide (Suliburk et al., 2009). A study showed that S-KET induced human HepG2
79 cell apoptosis through activating a Bax-mitochondria-caspase protease pathway (Lee
80 et al., 2009). However, the effect of S-KET on liver injury and oxidative stress as well

81 as its mechanisms remain fully elucidated.

82 Carbon tetrachloride (CCl₄)-induced hepatic damage is widely used as an
83 experimental model of human liver diseases and to identify potential hepatoprotective
84 agents. It is well established that targeting the Nrf2/HO-1 signaling pathway can
85 protect the liver from CCl₄-induced damage(Peng et al., 2018). In addition, it has been
86 confirmed that KET-induced up-regulation of the Nrf2/HO-1 signaling pathway
87 conveys gastroprotective and hepatoprotective effects.

88 Although KET has various beneficial effects, the protective effects, especially
89 those of S-KET, against CCl₄-liver injury and the associated underlying mechanisms
90 remain poorly understood. Therefore, based on these data, the aims of the present
91 study were to determine whether S-KET can protect the liver against CCl₄-induced
92 injury and, if so, to identify the potential underlying mechanisms, with a specific
93 focus on the Nrf2/HO-1 pathway.

94 **Materials and methods**

95 *Materials*

96 S-KET (purity > 99%) was purchased from Shanghai Aladdin Bio-Chem
97 Technology Co., Ltd. (Shanghai, China). CCl₄ was obtained from Sigma-Aldrich
98 Shanghai Trading Co., Ltd. (Shanghai, China). Assay kits for the detection of
99 superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and
100 glutathione peroxidase (GSH-Px) were procured from Jiancheng Biotechnology Co.,

Ltd. (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for the detection of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Animals

Male Sprague Dawley rats (n = 50; age, 6 weeks) were obtained from the Laboratory Animal Research Center and housed under conventional conditions. The study protocol was approved by the local Animal Ethics Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

The rats were randomly allocated to one of the five following treatment groups (Figure 1A): group 1, control group - oral (i.p.) pre-administration of phosphate-buffered saline (PBS; 0.5 mL/100 g) followed by intraperitoneal (i.p.) injection of 2.0 mL/kg of corn oil solution on day 6; group 2, CCl₄-induced model - pre-administration of PBS (p.o.; 0.5 mL/100 g) all 7 days followed by i.p. injection of 2.0 mL/kg CCl₄ in a 50% corn oil solution (Xie et al., 2010); groups 3–5, S-KET treatment groups - pre-treatment with S-KET (i.p.; 10, 20, or 40 mg/kg, volume: 1 mL/kg, dissolved in 0.9% NaCl) (Velísková et al., 1990) for 7 days with i.p. injection of 2.0 mL/kg CCl₄ in a 50% corn oil solution on day 6. Whole blood samples were collected and serum was obtained by centrifugation and stored at -70°C within 24 h.

Measurement of Biochemical

Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB), and gamma-glutamyltransferase (γ -GT) were quantified using commercial ELISA kits (BD Biosciences Pharmingen, San Diego, CA, USA; R&D Systems), in accordance with the manufacturers' instructions.

Histopathology of liver

Livers were removed from the rats, fixed in 10% paraformaldehyde for 48 h, embedded in paraffin, and sectioned at a thickness of 5 μ m. The sections were stained with hematoxylin and eosin (H&E), and photomicrographs were obtained with a transmission electron microscope.

Measurement of oxidative stress

Liver tissues were harvested and homogenized with cold 50 mM potassium phosphate buffer (pH 7.0) at 24 h after CCl₄ challenge. The homogenate was centrifuged at 980 $\times g$ for 10 min. Afterward, the supernatant was collected for the detection of SOD, MDA, GSH, and GSH-PX using bicinchoninic acid assay kits in accordance with the manufacturer's recommendation and as previously described (Zhao et al., 2017).

Cell apoptosis assessment

Liver cell apoptosis was measured using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay as described previously (Zhang et al.,

2019) . Briefly, liver cells were collected, fixed in 4% paraformaldehyde (freshly prepared), and then incubated with 0.1% Triton X-100. At each step, the cells were washed three times with aseptic PBS. Finally, the cells were incubated and stained with the TUNEL detection kit. Apoptotic cells were counted under a fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Western blot assay

The expression patterns of proteins in the Nrf2/HO-1 signaling pathway were detected by western blot analysis. Briefly, liver cells were harvested, washed twice with PBS, and digested with radioimmunoprecipitation assay buffer. Cellular protein lysates were collected and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, which were blocked with 5% nonfat milk in Tris-buffered saline and then probed with primary antibodies against cleaved caspase-3, Bax, Bcl-2, Nrf2, HO-1, glutathione-S-transferase (GST), quinone oxidoreductase1 (NQO-1), ATF4, ATF6, Grp78 and GAPDH, as a housekeeping gene, at 4°C overnight followed by incubation with appropriate secondary antibodies. Finally, the antibody signals were detected with Chemistar™ High-sig ECL Western Blotting Substrate (Tanon Science & Technology Co., Ltd., Shanghai, China). Each sample was analyzed three times. Protein levels were quantified by density analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of inflammatory factors in serum

Briefly, liver samples were collected, homogenized, and the supernatant was collected by centrifugation and stored at -70°C within 24 h after CCl_4 treatment. The expression levels of inflammatory factors in liver homogenates (i.e., IL-1 β , IL-6, and TNF- α) were quantified using commercial ELISA kits (BD Biosciences Pharmingen; R&D Systems) in accordance with the manufacturers' instructions.

Statistics

All experiments were repeated at least 3 times and data were presented as mean $\pm$ SD. The experimental data were analyzed using SPSS software (Version 17.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA was implemented for comparison among multiple groups followed by Tukey's post hoc tests. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of S-KET on liver parameters

Several serum indicators (ALT, AST, ALP, TB, and γ -GT) are often quantitated to evaluate the severity of hepatocellular damage. In the present study, these biochemical markers were markedly elevated at 24 h after CCl_4 challenge, whereas the increases were obviously inhibited by S-KET pretreatment at both low and high doses in a dose-dependent manner (Figure 1B-F).

Effects of S-KET on morphological changes in the rat liver

The severity of CCl_4 -induced histologic changes to the rat liver was analyzed. As

shown by the results presented in Figure 2, there were no histological alterations to the lobular architecture, hepatic cells, visible central veins, or thin sinusoids in the control group. In contrast, CCl₄ treatment induced severe liver damage, as demonstrated by diffuse centrilobular necrosis associated with panlobular inflammation. However, pre-treatment with S-KET attenuated CCl₄-induced necrosis and inflammatory cell infiltration.

S-KET attenuated CCl₄-induced oxidative stress

To evaluate the severity of CCl₄-induced oxidative stress in the rat liver, the expression levels of related enzymes (MDA, SOD, GSH, and GSH-PX) were quantified using commercial assay kits. The results (Figure 3) showed that exposure to CCl₄ markedly increased MDA activity and decreased SOD GSH, and GSH-PX levels as compared to the control group, while these changes were reversed by pre-treatment with S-KET.

Effects of S-KET on apoptosis of hepatocytes

The TUNEL assay was conducted to investigate CCl₄-induced death of hepatocytes. As shown by the results presented in Figure 4A and B, the incidence of cell apoptosis was notably decreased in group 2 as compared to the control group. In addition, S-KET inhibited apoptosis of hepatocytes in a dose-dependent manner (10–40 mg/kg). Next, to confirm the potential of S-KET to inhibit apoptosis, the expression levels of the apoptosis-related proteins Bcl-2, Bax, and cleaved caspase-3 were quantified with the western blot assay. As shown in Figure 4C–F, CCl₄

treatment obviously increased the expression levels of Bax and cleaved caspase-3 (pro-apoptotic) and decreased that of Bcl-2 (anti-apoptotic). Meanwhile, S-KET reversed the expression changes induced by CCl₄. The western blot assay results were in agreement with the findings of the TUNEL assay, which confirmed that S-KET inhibited CCl₄-induced cell death and subsequent liver injury.

S-KET attenuated CCl₄-induced inflammation.

Serum levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 were assayed to investigate the effects of S-KET on CCl₄-induced hepatic damage. As shown in Figure 5, CCl₄-treated increased the expression levels of TNF- α , IL-1 β , and IL-6, while pretreatment with S-KET markedly inhibited these increases in a dose-dependent manner.

S-KET activated the Nrf2/HO-1 signaling pathway.

The expression levels of the antioxidant transcription regulator Nrf2 and its downstream markers and candidate markers of the UPR pathway were evaluated in liver homogenates with the western blot assay to explore the role of the Nrf2/HO-1 signaling pathway in CCl₄-induced hepatic damage and the potential mechanism of the protective effects of S-KET. As shown by the results presented in Figure 6A, CCl₄ induced a significant increase in ATF4, ATF6 and Grp78 levels, whilst S-KET treatment remarkably decreased the expression of ATF4, ATF6 and Grp78. Figure 6B revealed that Nrf2/HO-1 proteins levels were slightly higher in the CCl₄-treated groups than the control group, meanwhile S-KET pre-treatment enhanced expression

225 of the Nrf2/HO-1 proteins (Nrf2, HO-1, NQO-1 and GST) and protected the liver
226 against oxidative stress-induced injury.

227 **Discussions**

228 KET exhibits beneficial anesthetic, sedative, and analgesic effects. Moreover,
229 KET reportedly conveys anti-depressant effects for the treatment of mental health
230 disorders(Bartlett et al., 2016; du Jardin et al., 2016), including post-traumatic stress
231 disorder(Liriano et al., 2019; Feder et al., 2014) among others(Murrough et al., 2015).
232 Besides, KET has been shown to protect against inflammation-induced organ damage
233 via up-regulation of the Nrf2/HO-1 pathway(Yang et al., 2013; Aswoco et al., 2015) .
234 However, the effects and associated mechanisms of KET, especially S-KET, on
235 CCl₄-induced liver damage are poorly understood and have never been reported.
236 Hence, based on the beneficial effects observed in the present and previous reports on
237 the protective effects of KET against oxidant injury and the safety and efficacy of
238 S-KET both *in vivo* and in clinical studies, we conducted a series of experiments to
239 determine whether S-KET can protect the liver against CCl₄-induced damage and to
240 explore the underlying mechanisms.

241 In this study, a rat model of CCl₄-induced hepatic injury was used to assess the
242 protective effects of S-KET against liver damage by evaluating various serum
243 biochemical parameters (ALT, AST, ALP, TB, and γ -GT) and histopathological
244 analyses. The contents of these biochemical markers represent the degree of injury

245 and necrosis to the liver cell membrane, as a consequence of hepatic damage. In the
246 present study, these biochemical markers were markedly increased in the CCl₄-treated
247 groups, which indicated hepatic cell damage, whereas these increases were obviously
248 reduced and inhibited by S-KET pretreatment in a dose-dependent manner. The
249 histopathological results also showed that S-KET pretreatment apparently attenuated
250 the degree of CCl₄-induced necrosis and inflammatory cell infiltration, which is in
251 agreement the biochemical assay results.

252 Besides, CCl₄ is a well-known hepatotoxin used for studying hepatotoxicity
253 leading to pathogenesis of hepatic injury and CCl₄ has been routinely used to establish
254 rodent models of acute liver injury to identify potential underlying mechanisms,
255 including inflammatory responses, oxidative stress, and cell apoptosis in large body of
256 studies(Hoek and Pastorino, 2002; Peng et al., 2018; Cong et al., 2017). The
257 metabolites of CCl₄ produces CC13* free radical, and covalent binding of CC13* free
258 radicals with cell components provoke lipoprotein secretion inhibition, thereby
259 causing steatosis; while CC13* free radical reacts bind with oxygen lead to formation
260 of CCl₃-O O* that results in lipid peroxidation reaction, which results in unbalanced
261 calcium homeostasis, hepatocytes apoptosis and death, and liver damage (Boll et al.,
262 2001). In addition, the excessive Kupffer cells induced by CCl₄, once primed by
263 oxidants, contribute to the exacerbation of oxidative stress and initiation of Kupffer
264 cells activation, which release pro-inflammatory cytokines and liver injury (Kiso et
265 al., 2012; Ponmari et al., 2014). Therefore, in the present study, inflammatory

cytokines and lipid peroxidation, which are important indicators of the inflammatory response and oxidative stress, respectively, were assayed to evaluate the effects of S-KET against liver injury.

Inflammation is an important component in maintaining homeostasis, while an overwhelmed inflammatory reaction may be associated to imbalance of this homeostatic status, which may lead to tissue injury or organ dysfunction (Loix et al., 2011). Administration of KET suppressed inflammatory reaction by inhibiting levels of TNF- α , IL-1 β , and IL-6 in dose-dependent manners (Gundogdu et al., 2016a). A recent systematic review and meta-analysis have revealed that intraoperative administration of KET could attenuate the early postoperative IL-6 inflammatory reaction (Dale et al., 2012). Besides, it was reported that the levels of inflammatory cytokines including IL-6 and TNF- α were both downregulated after treatment of KET in all observation times (Asmoro et al., 2015). In the present study, we found that application of S-KET significantly reversed the CCl₄-induced increased TNF- α , IL-1 β , and IL-6 in dose-dependent manners, suggesting that S-KET inhibited CCl₄-induced inflammatory response.

Lipid peroxidation is an important indicator of oxidative stress. As a principal cause of CCl₄-induced acute hepatic damage, lipid peroxidation ultimately triggers MDA production. In addition, the antioxidant defense systems, which include non-enzymatic antioxidants (e.g., GSH and GSH-PX) and defense-related enzymes (e.g., SOD and CAT), play critical roles against oxidative injury (El-Beltagi and

287 Mohamed, 2013; Kwiecien et al., 2014) . The results of the present study
288 demonstrated that exposure to CCl₄ markedly altered the activities of these
289 oxidant-related enzymes, while S-KET alleviated these changes. Moreover, CCl₄
290 administration increased the levels of the inflammatory cytokines IL-1 β , TNF- α , and
291 IL-6 in the rat liver, which were effectively weakened by pretreatment with S-KET in
292 a dose-dependent manner. These results suggested that the inhibition of oxidative
293 stress and the anti-inflammation effects of S-KET may partly contribute to protection
294 against CCl₄-induced hepatic damage in rats.

295 It is well established that oxidative stress induces cell apoptosis (Carmody and
296 Cotter, 2000; Tan et al., 1998). The results of the present and many previous studies
297 have revealed that the administration of S-KET can inhibit CCl₄-induced oxidative
298 stress. Therefore, in order to further explore the mechanisms underlying the
299 preventive and protective antioxidant effects of S-KET against liver injury, the
300 apoptosis of hepatocytes and the expression levels of apoptosis-related proteins
301 (cleaved caspase-3, Bax, and Bcl) were assessed using a rat model of CCl₄-induced
302 hepatic injury. The results indicated that the liver cells of CCl₄-treated rats showed a
303 higher rate of apoptosis, while pre-treatment with S-KET significantly prevented cell
304 death in a dose-dependent manner. Meanwhile, activation of cleaved caspase-3 and
305 Bax, and reduced expression of Bcl-2 induced by CCl₄ was also reversed by S-KET,
306 indicating that S-KET inhibits CCl₄-induced death of liver cells by regulating the
307 expression levels of apoptosis-associated proteins.

Nrf2 is a transcription factor that regulates the expression of HO-1, which is a cyto-protective enzyme that is generally induced in cellular defense against oxidative stress. Hence, Nrf2 activation is important to inhibit oxidative stress and maintain cellular homeostasis. In general, the expression of HO-1 facilitated by Nrf2 conveys antioxidant effects. Many studies conducted over the past few decades have reported that the Nrf2/HO-1 pathway protects the liver from CCl₄-induced damage (Peng et al., 2018; Ibrahim et al., 2020). Collectively, these findings suggested that the Nrf2/HO-1 pathway might play a significant protective role by inhibiting oxidative stress. Based on these reports, we hypothesized that S-KET might prevent oxidative stress via up-regulation of the Nrf2/HO-1 pathway to prevent liver injury. Hence, western blot analysis was conducted to investigate the expression levels of Nrf2 and HO-1 to confirm our prediction. The results demonstrated that S-KET further activated the expression of Nrf2 and HO-1. Accumulating evidence suggested that S-KET prevents oxidative stress and liver injury via activation of the Nrf2/HO-1 pathway.

The results of the present and previous studies showed that S-KET alleviated CCl₄-induced liver damage by inhibiting oxidative stress and inflammatory cell infiltration via activation of the Nrf2/HO-1 pathway.

In conclusion, the main finding of the present study was that S-KET is a potential candidate for the treatment of oxidative stress-related liver injury through the up-regulation/activation of the Nrf2/HO-1 signaling pathway. In addition, the Nrf2/HO-1 signaling pathway may be a potential therapeutic target for CCl₄-induced

329 liver injury.

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331 Not applicable.

332 **Conflicts of interest**

333 The Authors declare that there is no conflict of interest.

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337 **Availability of data and material**

338 The data used to support the findings of this study are available from the
339 corresponding author upon request.

340

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476

477 **Figure legends**

478 **Figure 1: Effects of S-KET on serum levels of AST, ALT, ALP, TB, and γ -GT in**
479 **CCl₄-treated rats.** (A). Treatment scheme. (B–F) Serum levels of AST, ALT, ALP,
480 TB, and γ -GT. All experiments were repeated at least 3 times and data were presented
481 as mean $\pm$ SD.

482 S-KET, S-ketamine; ALT, alanine aminotransferase; AST,
483 aspartate aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin;
484 γ -GT, γ -glutamyltransferase

485

486 **Figure 2: Effects of S-KET on histological changes in the livers of CCl₄-treated**
487 **rats.**

488 Histopathologic changes to the liver as detected by H&E staining ($\times 400$) and
489 transmission electron microscopy (TEM). The red arrows point to the inflammatory
490 infiltration, and the black arrows point to a inflammatory white spot.

491 S-KET, S-ketamine

492

493 **Figure 3: Effects of S-KET on oxidative stress in the livers of CCl₄-treated rats.**

494 The levels of MDA (A), SOD (B), GSH (C), and GSH-PX (D) in liver were detected
 495 using commercial assay kits. All experiments were repeated at least 3 times and data
 496 were presented as mean $\pm$ SD. $^*p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. CCl₄.
 497 S-KET, S-ketamine; MDA, malondialdehyde; SOD, superoxide dismutase; GSH,
 498 glutathione; GSH-PX, glutathione peroxidase

499

500 **Figure 4: Effects of S-KET on liver cell apoptosis of CCl₄-treated rats.**

501 (A and B). TUNEL assay was performed to measure liver cell apoptosis. (C–F)
 502 Western blot assay results of Bax, Bcl-2 and cleaved capase3 protein in liver cells. All
 503 experiments were repeated at least 3 times and data were presented as mean $\pm$ SD. *p
 504 < 0.05 vs. control; $^{\#}p < 0.05$ vs. CCl₄.
 505 S-KET, S-ketamine

506

507 **Figure 5: Effects of S-KET on inflammation in the livers of CCl₄-treated rats.**

508 Serum levels of TNF- α , IL-1 β , and IL-6 by ELISA.
 509 All experiments were repeated at least 3 times and values are presented as the mean $\pm$
 510 standard deviation (n = 10/group). $^*p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. CCl₄.
 511 S-KET, S-ketamine; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6,
 512 interleukin-6

513

514 **Figure 6: Effects of S-KET on Nrf2/HO-1 signaling pathway in the livers of**

515 **CCl₄treated rats.**

516 Expression levels of Nrf2/HO-1 related proteins in livers as determined with the
517 western blot assay. All experiments were repeated at least 3 times and data were
518 presented as mean $\pm$ SD.

519 S-KET, S-ketamine; GST, glutathione-S-transferase; NQO-1, quinone
520 oxidoreductase1.

Draft

Figure 1: Effects of S-KET on serum levels of AST, ALT, ALP, TB, and γ -GT in CCl₄-treated rats. (A). Treatment scheme. (B–F) Serum levels of AST, ALT, ALP, TB, and γ -GT. S-KET, S-ketamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin; γ -GT, γ -glutamyltransferase

348x182mm (300 x 300 DPI)

Figure 2: Effects of S-KET on histological changes in the livers of CCl₄-treated rats. Histopathologic changes to the liver as detected by H&E staining (×400) and transmission electron microscopy (TEM). The red arrows point to the inflammatory infiltration, and the black arrows point to a inflammatory white spot.
S-KET, S-ketamine

152x75mm (300 x 300 DPI)

Figure 3: Effects of S-KET on histological changes in the livers of CCl₄-treated rats. The levels of MDA (A), SOD (B), GSH (C), and GSH-PX (D) in liver were detected using commercial assay kits. *p < 0.05 vs. control; #p < 0.05 vs. CCl₄. S-KET, S-ketamine; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; GSH-PX, glutathione peroxidase

233x175mm (300 x 300 DPI)

Figure 4: Effects of S-KET on liver cell viability of CCl₄-treated rats. (A and B). TUNEL assay was performed to measure liver cell viability. (C–F) Western blot assay results of Bax, Bcl-2 and cleaved capase3 protein in liver cells. *p < 0.05 vs. control; #p < 0.05 vs. CCl₄. S-KET, S-ketamine

368x273mm (300 x 300 DPI)

Figure 5: Effects of S-KET on inflammation in the livers of CCl₄-treated rats.
Serum levels of TNF- α , IL-1 β , and IL-6 by ELISA.
Values are presented as the mean $\pm$ standard deviation (n = 10/group). *p < 0.05 vs. control; #p < 0.05 vs. CCl₄.
S-KET, S-ketamine; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6

332x86mm (300 x 300 DPI)

Figure 6: Effects of S-KET on Nrf2/HO-1 signaling pathway in the livers of CCl₄ treated rats. Expression levels of Nrf2/HO-1 related proteins in livers as determined with the western blot assay. All experiments were repeated at least 3 times and data were presented as mean $\pm$ SD. S-KET, S-ketamine; GST, glutathione-S-transferase; NQO-1, quinone oxidoreductase1.

286x204mm (300 x 300 DPI)