SUPPORTING INFORMATION

Kaempferol Prevents Acetaminophen-induced Liver Injury by Suppressing Hepatocyte Ferroptosis via Nrf2 Pathway Activation

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1. Materials and methods

1.1 Chemicals and reagents

The primary antibody specific against CD68 was purchased from Boster (Wuhan, China). The primary antibody specific against Keap1 was purchased from Affinity (Jiangsu, China).

1.2 Experimental design

Balb/c male mice, fasted for 22 hours, were injected with APAP (250 mg/kg) intraperitoneally (i.p.) and intragastrically administrated with KA (80 mg/kg, dissolved in 0.5% CMC-Na) or 0.5% CMC-Na 2 hours before APAP injection. The mice were anesthetized and sacrificed at 0, 1.5, 3 or 24 h after APAP treatment and the livers were immediately collected, frozen in liquid nitrogen and then stored at −80 °C until analysis.

1.3 Survival study

The survival study was performed as described previously[1]. Balb/c male mice were randomly divided into the APAP group and the KA treatment group. Mice were fasted for 22 hours, and intraperitoneally (i.p.) injected with APAP (300 mg/kg). The KA treatment group was intragastrically administrated with KA (80 mg/kg, dissolved in 0.5% CMC-Na) 2 hours before APAP injection, and the APAP group was intragastrically administrated with the same volume of 0.5% CMC-Na. The survival of the mice was observed and recorded over a 72-hour period.

1.4 Immunohistochemistry staining

Immunohistochemistry staining was performed as described previously[2]. Briefly, liver specimens fixed in 10% neutral buffered were embedded in paraffin blocks. For immunohistochemical analyses, liver sections (4 μm thick) were separated, rehydrated, and sequentially incubated with primary antibodies and secondary antibodies.

1.5 Measurement of hepatic GSH levels

For hepatic biochemical parameters determination, liver tissue was homogenized in nine-fold (g/mL) of ice-cold PBS, and then centrifuged at 600 ×g for 10 min at 4°C to obtain the supernatants[3]. Hepatic GSH levels were measured using reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by standard enzymatic procedures.

1.6 CCK8 assay

The L02 cells viability was measured by cell CCK8 colorimetric assay, according to the manufacturer's instructions (BioSharp, China). The CCK8 assay was performed as described previously[4]. L02 cells were subcultured in 96-well plates with complete medium and treated with different concentrations of KA for 24 h. After treatments, CCK8 reagent was added into each well, followed by incubating for another 1.5 h in a 37 ℃ incubator with 5% CO2. Then, the absorbance was measured by a microplate reader at 450 nm (Bio-Rad, Hercules, CA, USA), and cell viability was expressed as percentage values, as compared with the control group.

1.7 Quantitative real-time PCR (q-PCR)

Mouse liver RNA was extracted by TransZol Up Reagent, according to the manufacturer's instructions (TransGen, Beijing, China). Total RNA concentration was quantified by the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA). RNA was reverse transcribed using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) according to the manufacturer's instruction. cDNA was synthesized with the SYBR Green Supermix (Bio-Rad, Hercules, USA) and detected by LightCycler 480II (Roche, Indiana, USA). The sample was performed for 5 min at 95 ℃, 45 cycles of 10 s at 95 ℃ and 15 s at 60 ℃ and 20 s at 72 ℃. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change difference between experimental and calibration

samples[5]. *Gapdh* was used as an internal control. The primer sequences were summarized in Table S1.

1.8 Western blotting

Liver tissue and the L02 cells were lysed with RIPA buffer[6]. Proteins were subjected to SDS-PAGE electrophoresis and transferred to a PVDF membrane. After incubation in blocking buffer (5% nonfat milk in Tris-buffered saline Tween), membranes were incubated with specific antibodies for immunoblot analyses. Bands were visualized by the enhanced chemiluminescence (ECL) system (Clinx, Shanghai, China).

1.9 Statistical analysis

All data were statistically analyzed by using GraphPad Prism (version 6.0 for Windows), and values were expressed as the mean ± standard deviation (SD). Differences between the means of the two groups were analyzed by a two-tailed unpaired Student's t-test and were considered statistically significant when $P < 0.05$, while for comparisons between more than two groups a one-way ANOVA was performed.

2. Supplementary data

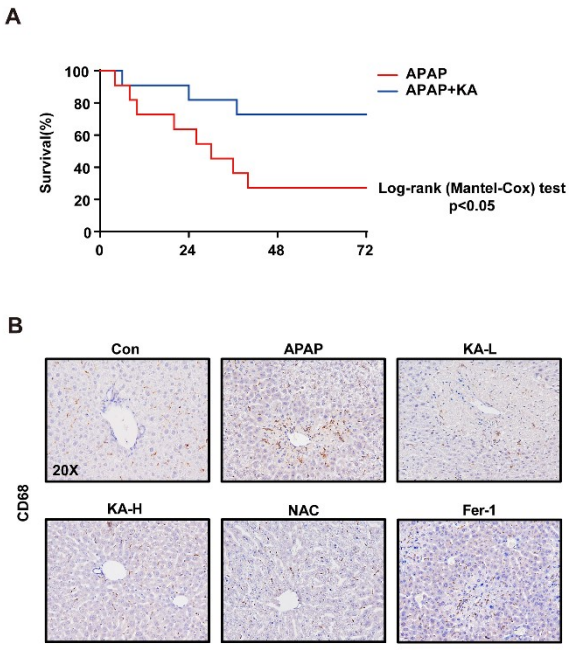

Figure S1. KA attenuates APAP-induced liver injury and inflammation.

(A) Balb/c male mice were fasted for 22 hours, and intraperitoneally (i.p.) injected with APAP (300 mg/kg). The KA treatment group was intragastrically administrated with KA (80 mg/kg, dissolved in 0.5% CMC-Na) 2 hours before APAP injection. The survival of the mice in the two groups was observed and recorded (n=10-11 per group). (B) Representative images of immunohistochemistry staining of hepatic CD68 in mice.

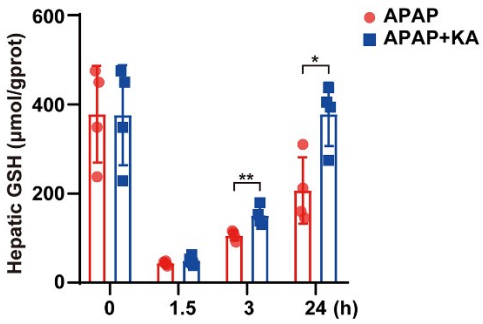

Figure S2. KA significantly reverses the decreased GSH levels induced by overdose APAP.

Balb/c male mice, fasted for 22 hours, were injected with APAP (250 mg/kg) intraperitoneally and treated with KA (80 mg/kg) or CMC-Na 2 hours before APAP injection. The mice were humanely sacrificed at 0, 1.5, 3 or 24 h after APAP treatment and the livers were collected. GSH levels of livers at different time after APAP injection were measured (n = 4 per group). The data are presented as the mean ± SD. *indicates a significant difference compared to the corresponding APAP group ; *$P < 0.05$, **$P < 0.01$.

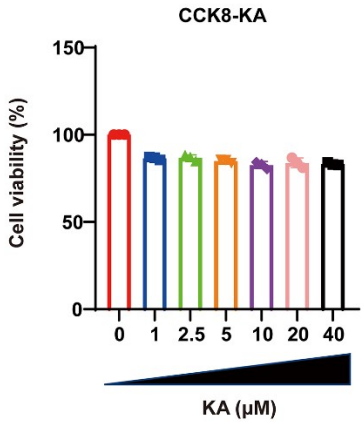

Figure S3. KA has no inhibitory effect on L02 cell activity.

Cell viabilities of L02 cells were examined by CCK8 assays after the treatment of different concentrations of KA (n = 3 per group).

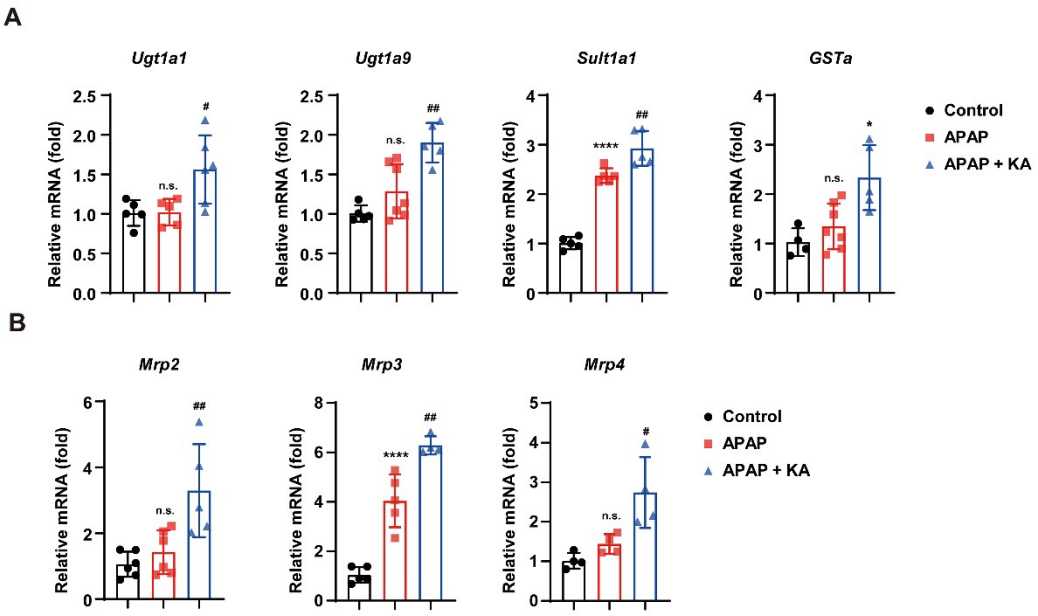

Figure S4. KA promotes the metabolism of APAP through two-phase metabolic pathways.

Balb/c male mice, fasted for 22 hours, were injected with APAP (250 mg/kg) intraperitoneally and treated with KA (80 mg/kg) and CMC-Na 2 hours before APAP injection. The mice were humanely sacrificed 24 h after APAP treatment. (A) qPCR analysis of the transcript levels of genes related to two-phase metabolism and detoxification of APAP (*Ugt1a1*, *Ugt1a9*, *Sult1a1* and *Gsta*). Gene expression was normalized to *Gapdh* mRNA levels (n=4-6 per group). (B) qPCR analysis of the transcript levels of genes related to multidrug resistance-associated proteins (*Mrp2*, *Mrp3* and *Mrp4*). Gene expression was normalized to Gapdh mRNA levels (n=4-6 per group). The data are presented as the mean ± SD. * indicates a significant difference compared to the control group; $^{****}P < 0.0001$. # indicates a significant difference compared to the APAP group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

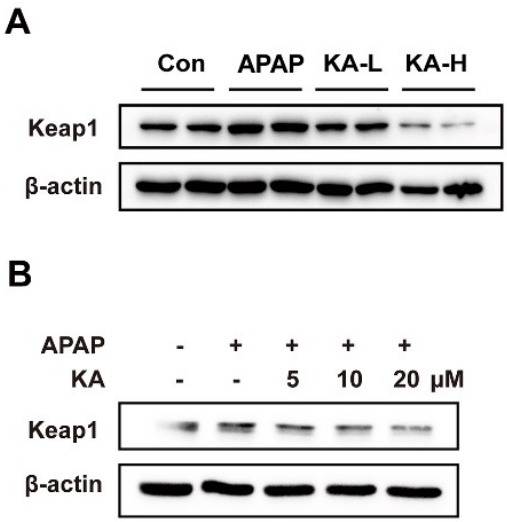

Figure S5. KA reduces Keap1 protein levels increased by APAP *in vivo* and *in vitro*.

(A) The Keap1 expression in mouse liver was detected by immunoblotting using a specific antibody, and β-actin was used as a loading control. (B) L02 cells were treated with KA (0 μM, 5 μM, 10 μM or 20 μM) in response to APAP (5 mM) for 24 h. The protein expression of Keap1 was detected by immunoblotting, and β-actin was used as a loading control.

Table S1. Primer sequences for real-time PCR

Primer name	Forward primer sequence	Reverse primer sequence
Human		
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
PTGS2	CGGTGAAACTCTGGCTAGACAG	GCAAACCGTAGATGCTCAGGGA
NRF2	TCCAGTCAGAAACCAGTGGAT	GAATGTCTGCGCCAAAAGCTG
GCLC	GCAAGGCCCAGAACAGCACG	TCCCTCATCCATCTGGCAACTGT
SOD2	CCCGACCTGCCCTACGACTAC	AACGCCTCCTGGTACTTCTCCTC
NOX2	AAGATGCGTGGAAACTACCTAA	TTTTTGAGCTTCAGATTGGTGG
Mice		
Gapdh	AGGAGTAAGAAACCCTGGAC	CTGGGATGGAATTGTGAG
Ptgs2	ATTCCAAACCAGCAGACTCATA	CTTGAGTTTGAAGTGGTAACCG
Gpx4	GCCAAAGTCCTAGGAAACGC	CCGGGTTGAAAGGTTCAGGA
Nrf2	GAGACGGCCATGACTGAT	GTGAGGGGATCGATGAGTAA
Tnfa	TAGGCCATTGTGTATGCAGC	ACTTCCAACCCAGGTCCTTC
Il1b	TCAGGACAGATGCAGATGCT	CTGGAAAACCTTCCTGCTGT
Il6	TAGGCCATTGTGTATGCAGC	ACATGTTCAGCTTTGTGGACC
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTCGGGT
Ccl5	GTATTTCTACACCAGCAGCAAG	TCTTGAACCCACTTCTTCTCTG
Acsl4	CTCACCATTATATTGCTGCCTGT	TCTCTTTGCCATAGCGTTTTTCT
Sod2	TCCCAGACCTGCCTTACGACTAT	GGTGGCGTTGAGATTGTTCA
Ugt1a1	GCTTCTTCCGTACCTTCTGTTG	GCTGCTGAATAACTCCAAGCAT
Ugt1a9	TGTGTGGATTAATTGTCGCCA	CAAAGATCACTGATGGGAGCG
Sult1a1	TGATCTATCAGGGTGGCAAGC	CAGAGTTTCAAGACCTGGGGG
Gsta	AAGCCCGTGCTTCACTACTTC	GGGCACTTGGTCAAACATCAAA
Mrp2	GTGTGGATTCCCTTGGGCTTT	CACAACGAACACCTGCTTGG
Mrp3	ACTTCCTCCGAAACTACGCACC	GCTGGCTCATTGTCTGTCAGGT
Mrp4	CATCGCGGTAACCGTCCTC	CCGCAGTTTTACTCCGCAG

References

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