Copper oxide nanoparticles induce cuproptosis and ferroptosis through mitochondrial concatenation

Muran Jiang[1], Xiaoqi Tao[1,2*], Yingxin Pang[1], Zongmin Qin[1], Erqun Song[3], Yang Song[4,*]

[1] Key Laboratory of Luminescence Analysis and Molecular Sensing, Ministry of Education, College of Food Science, Southwest University;

[2] Joint International Research Laboratory of Animal Health and Animal Food Safety, College of Veterinary Medicine, Southwest University;

[3] Key Laboratory of Luminescence Analysis and Molecular Sensing, Ministry of Education, College of Pharmaceutical Sciences, Southwest University;

[4] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences

*Corresponding Authors: X. T., Key Laboratory of Luminescence Analysis and Molecular Sensing, Ministry of Education, College of Food Science, Southwest University; Joint International Research Laboratory of Animal Health and Animal Food Safety, College of Veterinary Medicine, Southwest University, 2 Tiansheng Rd, Beibei District, Chongqing, 400715, China; E-mail: taoxiaoqi@swu.edu.cn; Y. S., State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Rd, Haidian District, Beijing, 100085, China; E-mail: yangsong@rcees.ac.cn

Gene	Description	Primer Sequence
β-actin	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT
DLAT	Forward	CTTTAGCCTCCAAAGCGAGAG
	Reverse	AGATTGTAAATGTTCCACCCTGG
LIAS	Forward	CCTGGGGTCCCGGATATTTG
	Reverse	GAAGGTCTGGTCCATTATGCAA
FDX1	Forward	CAAGGGGAAAATTGGCGACTC
	Reverse	TTGGTCAGACAAACTTGGCAG
HSP70	Forward	GAGATCGACTCTCTGTTCGAGG
	Reverse	GCCCGTTGAAGAAGTCCTG
TFR	Forward	GTTTCTGCCAGCCCCTTATTAT
	Reverse	GCAAGGAAAGGATATGCAGCA
FPN	Forward	ACCAAGGCAAGAGATCAAACC
	Reverse	AGACACTGCAAAGTGCCACAT
FTH	Forward	CAAGTGCGCCAGAACTACCA
	Reverse	GCCACATCATCTCGGTCAAAA
GPX4	Forward	GCCTGGATAAGTACAGGGGTT
	Reverse	CATGCAGATCGACTAGCTGAG
ACC2	Forward	CGCTCACCAACAGTAAGGTGG
	Reverse	GCTTGGCAGGGAGTTCCTC
SCD1	Forward	TTCTTGCGATACACTCTGGTGC
	Reverse	CGGGATTGAATGTTCTTGTCGT
ACLY	Forward	ACCCTTTCACTGGGGATCACA
	Reverse	GACAGGGATCAGGATTTCCTTG
SCAP	Forward	TGGAGCTTTTGAGACTCAGGA
	Reverse	TCGATTAAGCAGGTGAGGTCG
FASN	Forward	GGAGGTGGTGATAGCCGGTAT
	Reverse	TGGGTAATCCATAGAGCCCAG
IL-10	Forward	GCTCTTACTGACTGGCATGAG
	Reverse	CGCAGCTCTAGGAGCATGTG
IL-6	Forward	TAGTCCTTCCTACCCCAATTTCC
	Reverse	TTGGTCCTTAGCCACTCCTTC
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCCGAAGTTCAGTAG

Table S1. RT-qPCR primer pairs used in this study

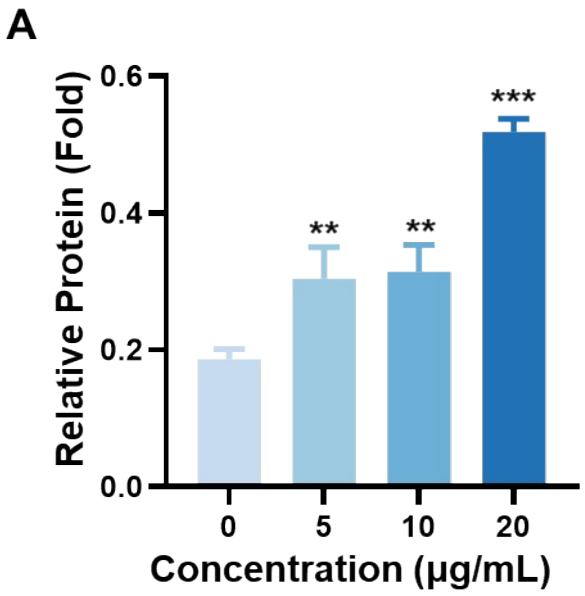

Figure S1. Expression and oligomerization of Lip-DLAT protein in RAW264.7 cells with Image J software (A). Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control. n*s* indicates no significance.

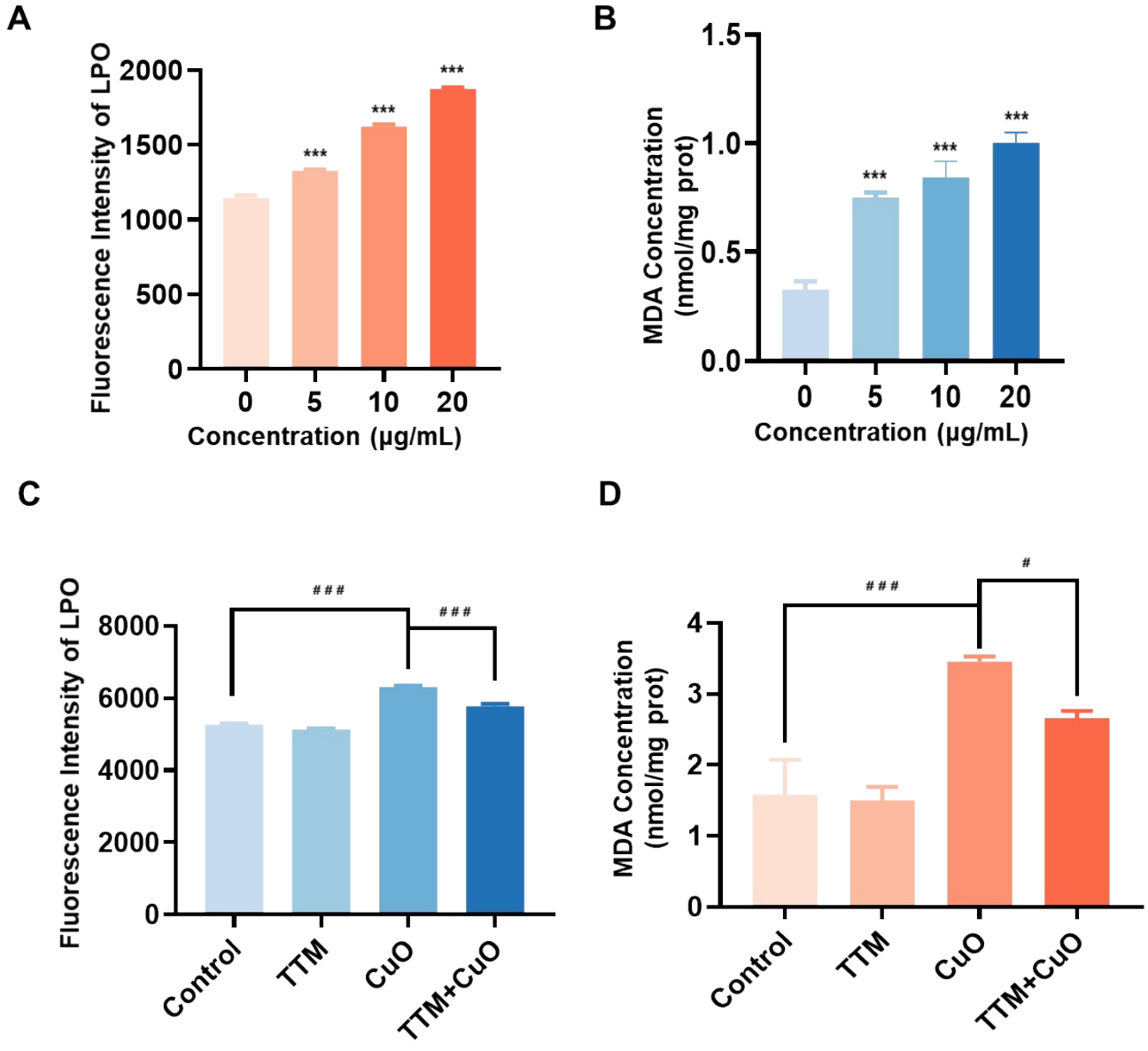

Figure S2. Following 12 h treatment of RAW264.7 cells with varying concentrations of CuO NPs (0, 5, 10, 20 μg/mL), intracellular levels of lipid peroxidation (A) and MDA (B) content were quantified. RAW264.7 cells were pre-incubated with 100 μM TTM for 1 h at 37℃, after which they were subjected to 20 μg/mL CuO NPs for 12 h. Subsequent analyses included the evaluation of intracellular lipid peroxidation levels (C) and the intracellular MDA content (D). Data are presented as mean ± SD. $^{***}P < 0.001$ compared to control. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to 20 μg/mL CuO.

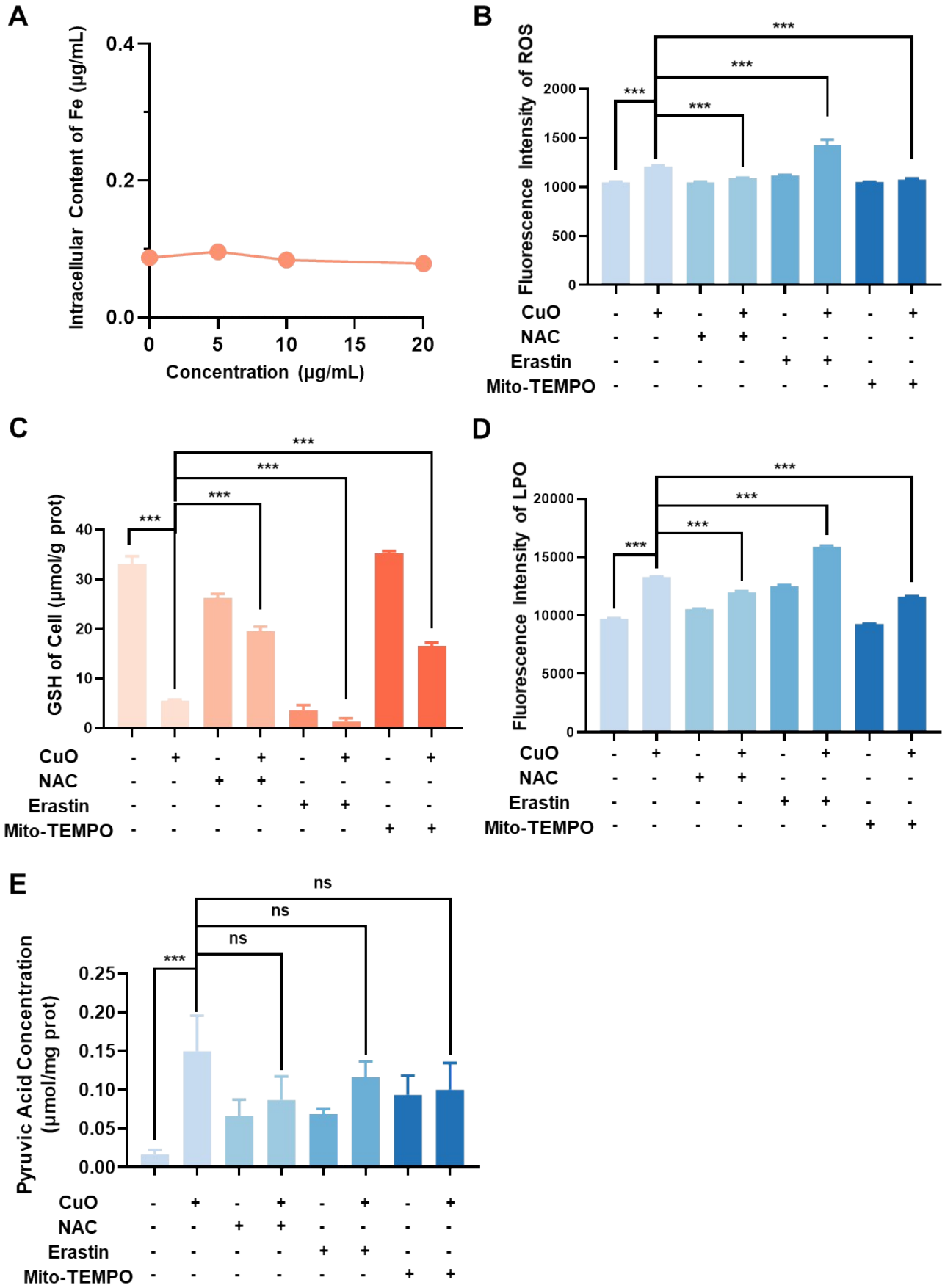
A
Intracellular Content of Fe (μg/mL)
0.4
0.2
0.0
0
5
10
15
20
Concentration (μg/mL)
B
Fluorescence Intensity of ROS
2000
1500
1000
500
0
***
***
***
CuO
NAC
Erastin
Mito-TEMPO
C
GSH of Cell (μmol/g prot)
40
30
20
10
0
***
***
***
***
CuO
NAC
Erastin
Mito-TEMPO
D
Fluorescence Intensity of LPO
20000
15000
10000
5000
0
***
***
***
***
CuO
NAC
Erastin
Mito-TEMPO
E
Pyruvic Acid Concentration (μmol/mg prot)
0.25
0.20
0.15
0.10
0.05
0.00
***
ns
ns
ns
CuO
NAC
Erastin
Mito-TEMPO

Figure S3. RAW264.7 cells were exposed to various concentrations of CuO NPs for 12 h, and the intracellular iron content was quantified using ICP-OES (A). RAW264.7 cells were co-incubated with 10 mM NAC, 10 μM Erastin, and 10 μM Mito-TEMPO for 1 h, and then exposed to 20 μg/mL CuO NPs for 12 h to assess intracellular ROS (B) and GSH (C) levels, intracellular lipid peroxidation (D) and pyruvic acid content (E). ***$P < 0.001$ compared to 20 μg/mL CuO. *ns* indicates no significance.

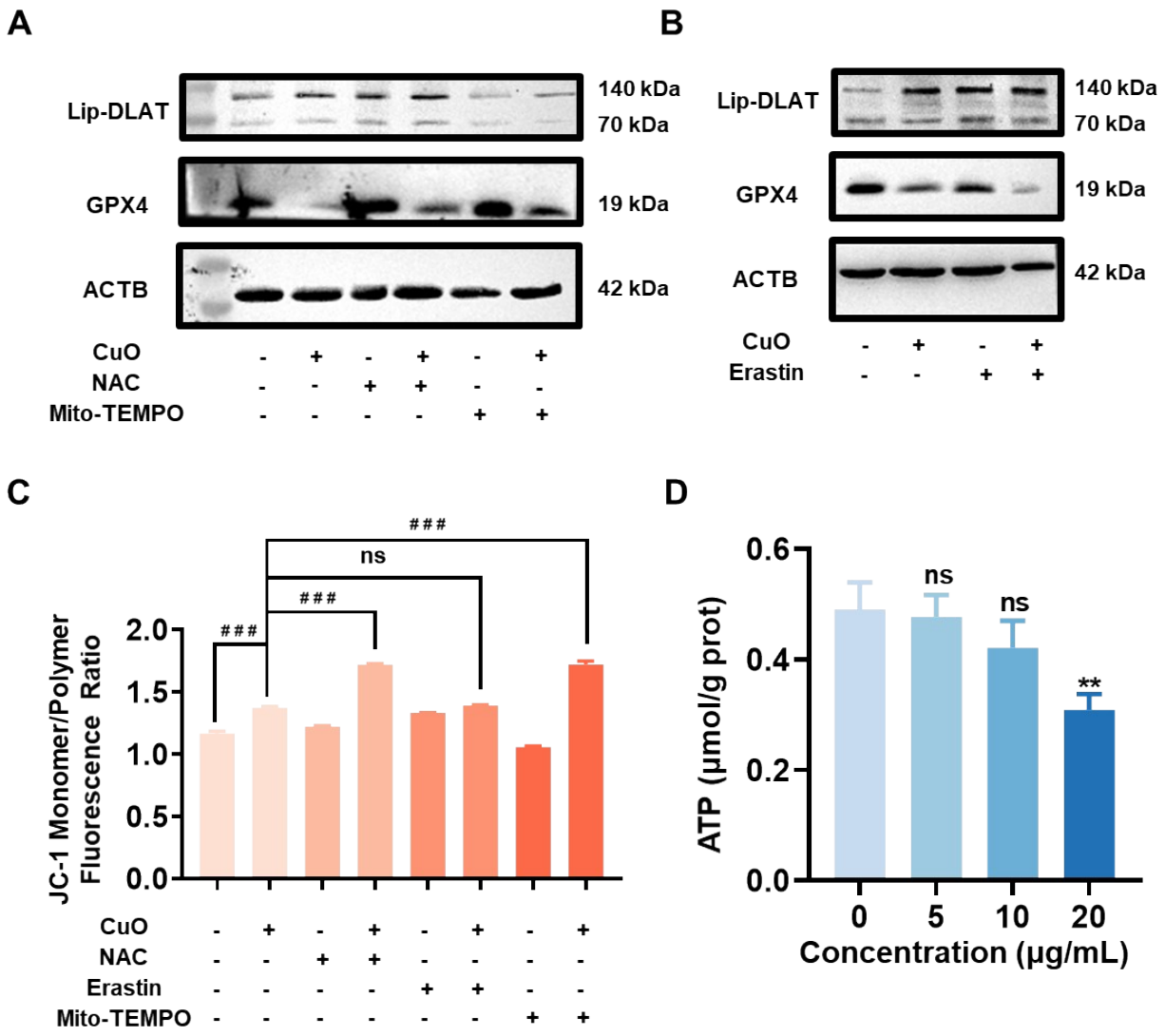

Figure S4. Western blotting was employed to analyze the oligomerization of Lip-DLAT and the expression of GPX4 in RAW264.7 cells that were pre-treated with 10 mM NAC and 10 μM Mito-TEMPO (A), as well as 10 μM Erastin (B) for 1 h, before being exposed to 20 μg/mL CuO NPs for 12 h. In the same experimental setup, the JC-1 mitochondrial membrane potential assay kit was utilized to evaluate the tochondrial membrane potential in RAW264.7 cells (C). Following exposure to various concentrations of CuO NPs for 12 h, the intracellular ATP content in RAW264.7 cells was quantified (D). Data are presented as mean ± SD. $^{**}P < 0.01$ compared to control. $^{###}P < 0.001$ compared to 20 μg/mL CuO. *ns* indicates no significance.

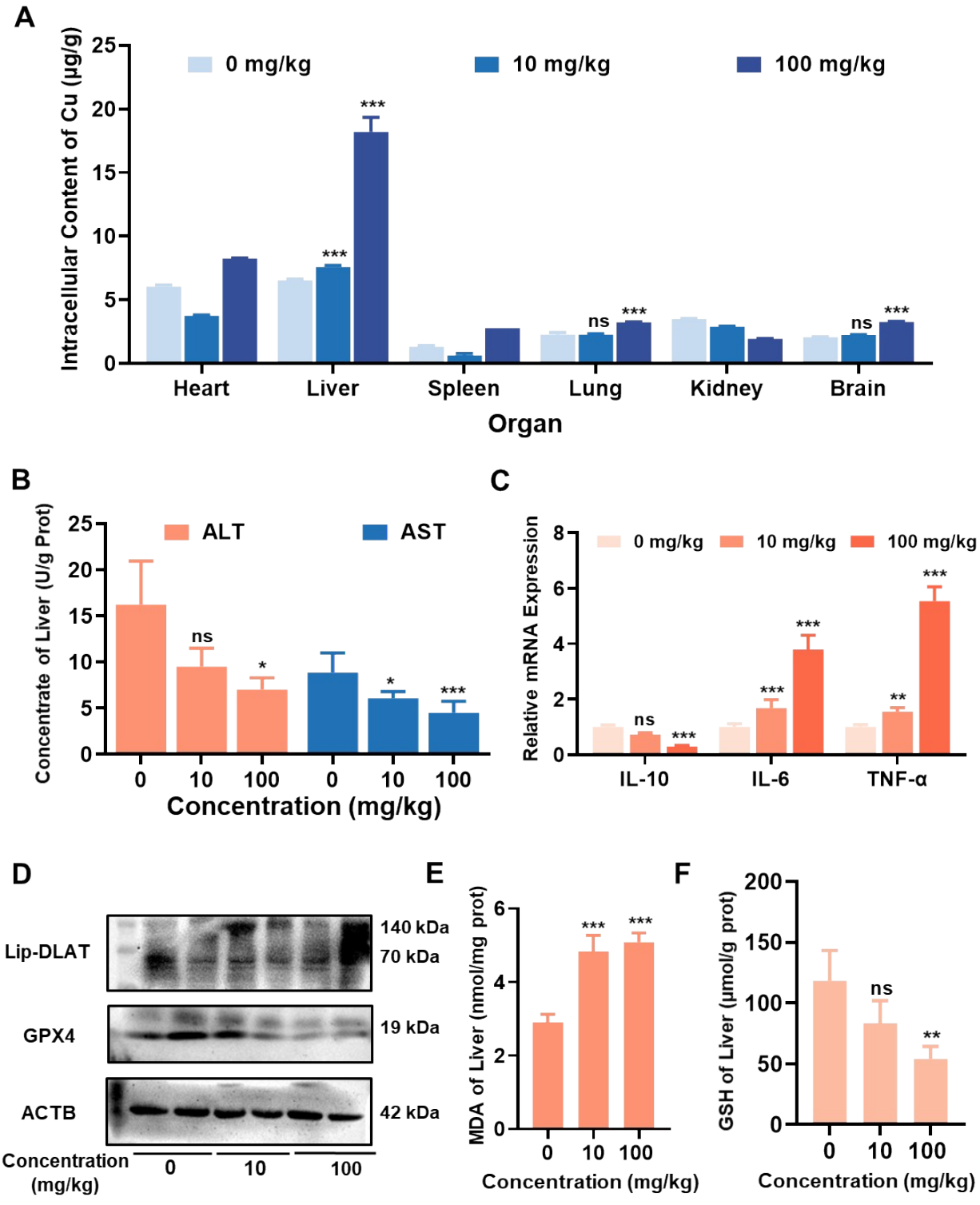

Figure S5. Male C57BL/6 mice, aged 6-8 weeks, were orally administered with doses of 0, 10, and 100 mg/kg of CuO NPs for 35 days. Organs, including the heart, liver, spleen, lungs, kidneys, and brain, were collected and digested for the assessment of copper element accumulation using ICP-OES (A). The content of ALT and AST in the liver (B). The mRNA expression levels of inflammatory

factors IL-10, IL-6, and TNF-α, the aggregation status of protein Lip-DLAT and the expression level of GPX4 (D) in the mouse liver. The levels of MDA (E) and GSH (F) in the liver. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to Control. *ns* indicates no significance.

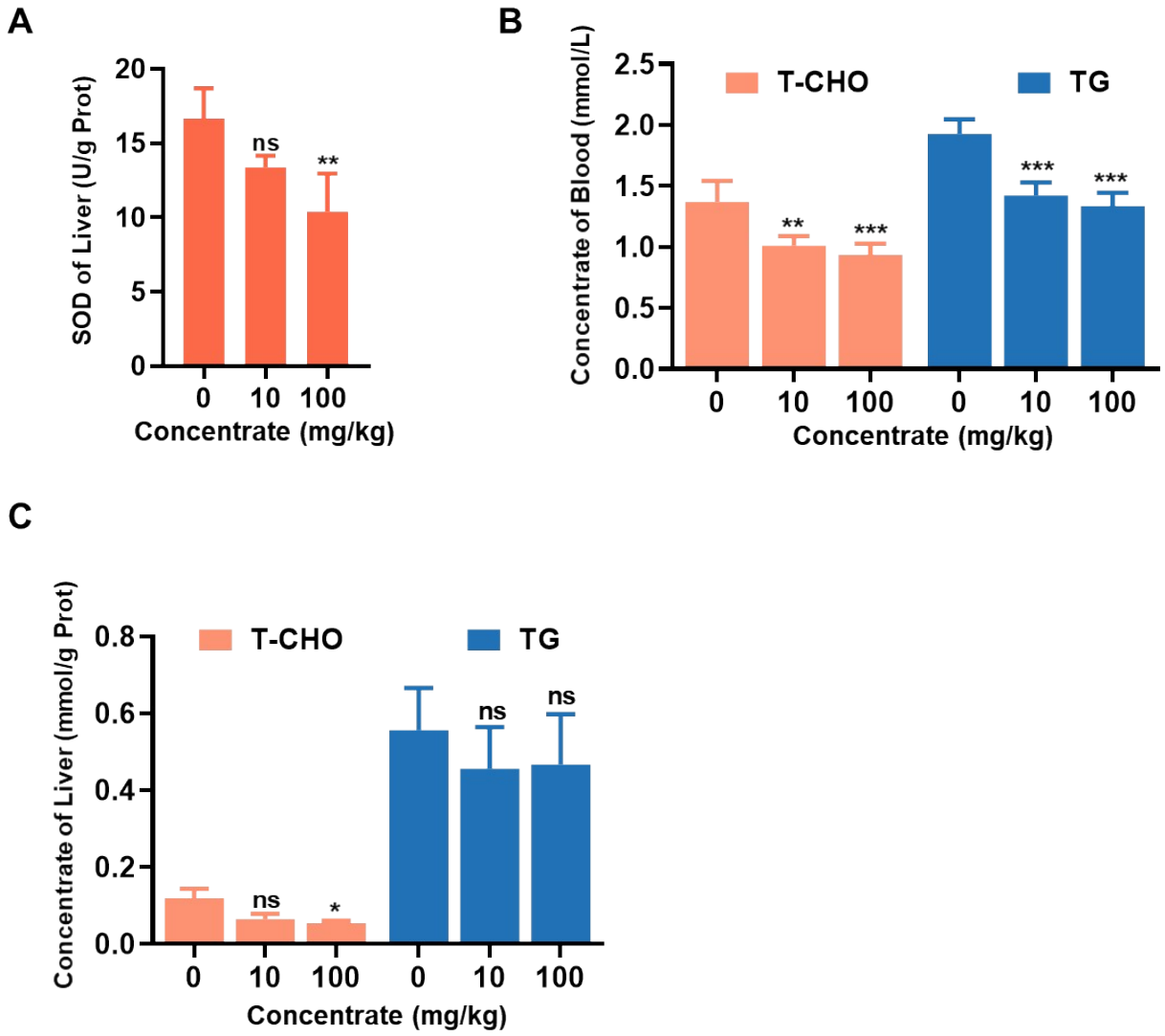

Figure S6. Male C57BL/6 mice, aged 6-8 weeks, were orally administered with doses of 0, 10, and 100 mg/kg of CuO NPs for 35 days. The level of SOD in the liver (A). The content of T-CHO and TC in the plasma (B) and liver (C). Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to Control. *ns* indicates no significance.