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Supplementary Information

Toxico-metabolomics study of a deep eutectic solvent comprising choline chloride and urea suggests in vivo toxicity involving oxidative stress and ammonia stress

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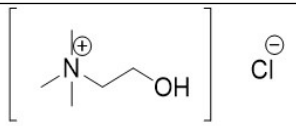
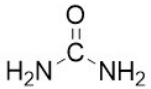
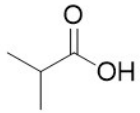
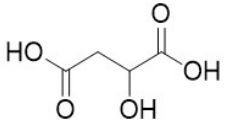
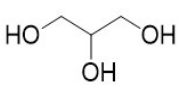
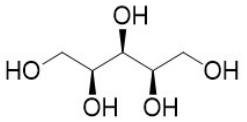
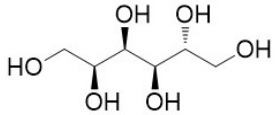
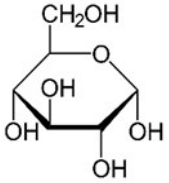
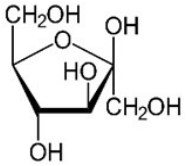
S1. Chemicals and reagents

Chemicals and reagents used in this study are listed in Table S1, where chemical structures are provided for DES components.

Table S1. Chemicals and reagents used in the present study.

Name (% purity)	Source	Note	
Choline chloride (ChCl; ≥ 98%)	Sigma-Aldrich (St. Louis, MO, USA)	DES components	
Urea (≥ 98%)			
Lactic acid (≥ 98%)			
Malic acid (≥ 98%)			
Glycerol (≥ 99.5%)			

Xylitol (≥ 99%)		
Sorbitol (≥ 99.5%)		
Glucose (≥ 99.5%)		
Fructose (≥ 99%)		
DL-alanine-d_4 (≥ 99%)	Sigma-Aldrich	Internal standards
Stearic acid-d_{35} (≥ 99%)		
Ribitol (≥ 99%)		
4-Chloro-DL-phenylalanine		
Chlorpropamide (≥ 97%)		
Methoxyamine hydrochloride (≥ 98%)	Sigma-Aldrich	Derivatization agents
N,O-Bis(trimethylsilyl)trifluoroacetamide with		

trimethylchlorosilane (BSTFA + TMCS, 99:1)		
Chloroform (≥ 99.9%)	Sigma-Aldrich	
Acetic acid (≥ 99.7%)		
Formic acid (> 99%)	Thermo-Fisher Scientific	
	(Rockford, IL, USA)	
Methanol	J.T. Baker	HPLC-grade
Acetonitrile	(Center Valley, PA, USA)	
Water		
0.25% trypsin-EDTA (TE)	Thermo-Fisher Scientific	Cell culture
Fetal bovine serum (FBS)		
Dulbecco's Modified Eagle's Medium (DMEM)	HyClone Laboratories Inc.	HepG2 culture medium
Roswell Park Memorial Institute 1640 Medium	(Logan, UT, USA)	HEK293T culture medium
(RPMI-1640)		
Dimethyl sulfoxide (DMSO, >99%)	Samchun	Cytotoxicity assay
	(Pyeongtaek, Korea)	
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich	

ab65354 (Superoxide dismutase assay) ab83464 (Catalase assay) ab118970 (Malondialdehyde assay) ab205811 (Ratio of glutathione to glutathione disulfide assay)	Abcam (Cambridge, MA, USA)	Oxidative stress assay
AA0100 (Ammonia assay)	Sigma-Aldrich	Ammonia assay

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S2. Cytotoxicity assay procedures and results

CL (1:1), CL (1:1) (aq), CM (1:1), CM (1:1) (aq), lactic acid, and malic acid were tested at 100, 500, 1,000, 2,500, 5,000, 8,000, and 10,000 μg mL^{-1}. The other species were tested at 1,000, 2,000, 8,000, 10,000, 20,000, 40,000, and 80,000 μg mL^{-1}. The tested concentrations in mM are listed in Table S2. The resulting IC_{50} values are listed in Tables S3 and S4 for HepG2 and HEK293T, respectively.

Table S2. Tested concentrations of DESs and associated species to determine IC_{50} values.

Testing species	Tested concentration							
	μg mL^{-1}	100	500	1,000	2,500	5,000	8,000	10,000
CL (1:1) / CL (1:1) (aq)	mM	0.44	2.18	4.35	10.9	21.8	34.8	43.5
CM (1:1) / CM (1:1) (aq)		0.37	1.83	3.65	9.13	18.3	29.2	36.5
Lactic acid		1.11	5.55	11.1	27.8	55.5	88.8	111
Malic acid		0.75	3.73	7.46	18.6	37.3	59.7	74.6
	μg mL^{-1}	1,000	2,000	8,000	10,000	20,000	40,000	80,000
CG (1:2) / CG (1:2) (aq)	mM	3.09	6.18	24.7	31.0	61.8	124	247
CX (1:1) / CX (1:1) (aq)		3.43	6.85	27.4	34.3	68.5	137	274
CS (1:1) / CS (1:1) (aq)		3.11	6.22	24.9	31.1	62.2	124	249
CGL (5:2) / CGL (5:2) (aq)		0.87	1.74	6.97	8.71	17.4	34.8	69.7
CF (5:2) / CF (5:2) (aq)		0.87	1.74	6.97	8.71	17.4	34.8	69.7
CU (1:2) / CU (1:2) (aq)		3.85	7.7	30.8	38.5	77.0	154	308
Glycerol		10.9	21.7	86.9	109	217	434	869
Xylitol		6.57	13.1	52.6	65.7	131	263	526
Sorbitol		5.49	11.0	43.9	54.9	110	220	439

Glucose	5.55	11.1	44.4	55.5	111	222	444
Fructose	5.55	11.1	44.4	55.5	111	222	444
Urea	16.6	33.3	133	166	333	666	1,331
ChCl	7.16	14.3	57.3	71.6	143	287	573

Table S3. IC_{50} values (mean ± SD; n=10) of the tested species for HepG2 cell line.

DES component	IC_{50} (mM)		DES	IC_{50} (mM)		DES (aq)	IC_{50} (mM)	
ChCl	43.5	(± 6.1)						
Lactic acid	20.1	(± 1.9)	CL (1:1)	15.2	(± 1.3)	CL (1:1) (aq)	15.0	(± 0.9)
Malic acid	11.2	(± 1.5)	CM (1:1)	8.8	(± 1.5)	CM (1:1) (aq)	9.5	(± 0.3)
Glycerol	> 869		CG (1:2)	33.3	(± 8.3)	CG (1:2) (aq)	33.6.	(± 7.1)
Xylitol	> 526		CX (1:1) [a]	19.5	(± 5.8)	CX (1:1) (aq) [a]	69.4.	(± 18.3)
Sorbitol	243	(± 59)	CS (1:1) [b]	18.0	(± 8.3)	CS (1:1) (aq) [b]	54.8	(± 10.8)
Glucose	293	(± 106)	CGL (5:2)	9.1	(± 4.0)	CGL (5:2) (aq)	9.4	(± 1.1)
Fructose	247	(± 19)	CF (5:2) [c]	5.1	(± 2.1)	CF (5:2) (aq) [c]	10.9	(± 5.9)
Urea	419	(± 41)	CU (1:2) [d]	28.4	(± 7.7)	CU (1:2) (aq) [d]	49.0	(±7.4)

[a] $p = 0.0002$ between DES and DES (aq).

[b] $p = 0.0003$ between DES and DES (aq).

[c] $p = 0.0204$ between DES and DES (aq).

[d] $p = 0.0186$ between DES and DES (aq).

Table S4. IC_{50} values (mean ± SD; n=10) of the tested species for HEK293T cell line.

DES component	IC_{50} (mM)		DES	IC_{50} (mM)		DES (aq)	IC_{50} (mM)	
ChCl	89.7	(±26.9)						
Lactic acid	14.0	(±0.5)	CL (1:1) [a]	13.0	(± 0.6)	CL (1:1) (aq) [a]	20.6	(±8.1)
Malic acid	65.0	(±11.8)	CM (1:1) [b]	38.7	(± 12.2)	CM (1:1) (aq) [b]	16.4	(±0.4)
Glycerol	777	(±57.5)	CG (1:2) [c]	74.5	(± 20.7)	CG (1:2) (aq) [c]	128	(±47)
Xylitol	> 526		CX (1:1)	160	(± 66.6)	CX (1:1) (aq)	107	(±46)
Sorbitol	371	(±61.5)	CS (1:1)	65.8	(± 17.6)	CS (1:1) (aq)	87.5	(±26.8)
Glucose	277	(±33.4)	CGL (5:2)	21.5	(± 8.7)	CGL (5:2) (aq)	28.1	(±11.9)
Fructose	303	(±22.8)	CF (5:2)	19.0	(± 9.1)	CF (5:2) (aq)	33.4	(±17.7)
Urea	541	(±53.0)	CU (1:2)	101	(± 31.1)	CU (1:2) (aq)	100	(±32)

[a] $p = 0.0194$ between DES and DES (aq).

[b] $p = 0.0002$ between DES and DES (aq).

[c] $p = 0.0281$ between DES and DES (aq).

S3. Detailed information on doses for in vivo study

The density of CU (1:2) is 1.19 g cm^{-3} [2]. Two-fold dilution of CU (1:2) with water results in 50% v/v CU (1:2). The % v/v concentration of this solution can be converted to % w/w concentration by the following calculation:

$$\left(50 \times 10^{-3}\, mL \times 1.19\, g\, mL^{-1}\right) \div \left(50 \times 10^{-3}\, mL \times 1.19\, g\, mL^{-1} + 50 \times 10^{-3}\, g\right) \times 100 = 54.3\%\, w/w$$ CU (1:2) or 45.7% w/w water

In in vivo study, CU (1:2) was administered using a water-diluted formulation (50% v/v CU). The administration dose of CU (1:2) equivalent to 1.5 g kg^{-1} was calculated using the average mouse weight of 28 g (see the footnote of Table S5 for calculation). Detailed dose information in the five groups is provided in Table S5.

Table S5. Doses and volumes of the testing species for administration in *in vivo* study.

Group	Treatment	Amount administered to each mouse			Volume of administration
		ChCl	Urea	Total	
Group i	Saline	- [a]	-	-	70.4 μL of saline
Group ii	CU (1:2)	22.6 mg (=0.162 mmol)	19.4 mg (=0.323 mmol)	42 mg [b]	70.4 μL of 50% v/v CU (1:2) [c] (CU (1:2):water, 1:1 v/v)
Group iii	CU (1:2) (aq)	22.6 mg (=0.162 mmol)	19.4 mg (=0.323 mmol)	42 mg	70.4 μL of aqueous solution containing 42 mg of ChCl and urea in total [d]
Group iv	ChCl	22.6 mg (=0.162 mmol)	-	22.6 mg	70.4 μL of aqueous solution containing 22.6 mg of ChCl [d]
Group v	Urea	-	19.4 mg (=0.323 mmol)	19.4 mg	70.4 μL of aqueous solution containing 19.4 mg of urea [d]

[a] None.

[b] $(42\ mg \times 10^{-3}\ mg\ g^{-1}\ CU\ (1{:}2)) \div (28\ g \times 10^{-3}\ g\ kg^{-1}\ mouse\ weight) = 1.5\ g\ kg^{-1}$.

[c] Equals to 54.3% w/w CU or 45.7% w/w water.

[d] No heat was applied to prepare the solution.

S4. Identified differential metabolites and heat-map presentation

The identified differential metabolites in serum, kidney, and liver are listed in Tables S6, S7, and S8, respectively. In the heat-map presentation of group ii (Figure 4a), eight out of 16 biological replicates were selected randomly and shown in the map only to have the same number of samples as the other groups. The pathway analysis results presented in Figures 4b-4d are listed in Table S9.

Table S6. A list of differential metabolites identified in serum.

Metabolite	t_R (min) [a]	Group ii (treated with CU (1:2)) *vs*. Group i (treated with saline)		
		VIP score	Fold change	t-test (*p*)
Pyruvate [b]	5.90	1.66	1.32	0.003
L-Lactic acid [b]	6.10	1.11	0.68	0.023
L-Alanine [b]	6.81	3.59	0.62	0.002
? [d]	7.14	1.72	0.94	0.015
Oxalic acid [b]	7.45	4.57	0.75	< 0.0001
L-Valine [b]	8.66	5.32	0.6	< 0.0001
Urea [b]	8.99	6.08	0.83	< 0.0001
L-Leucine [b]	9.52	5.22	0.52	< 0.0001
?	9.58	3.07	1.59	0.004
?	9.67	1.80	1.25	0.005
L-Isoleucine [b]	9.84	2.30	0.31	< 0.0001
L-Proline [b]	9.88	4.94	0.55	< 0.0001
L-Serine [b]	10.82	2.92	0.67	< 0.0001
L-Threonine [b]	11.2	3.00	0.64	< 0.0001
?	12.87	1.86	0.64	< 0.0001
Ornithine [b]	14.03	1.74	0.71	0.002
Phenylalanine [b]	14.18	2.04	0.69	< 0.0001
Taurine [b]	14.69	1.02	0.6	< 0.0001
L-Glutamic acid [b]	15.81	2.92	0.78	0.000
?	16.01	1.32	0.64	0.015

?	16.32	2.78	0.57	< 0.0001
Citric acid [b]	16.39	1.09	0.75	0.010
?	16.74	1.42	0.86	0.001
L-Tyrosine [b]	17.56	1.09	0.68	< 0.0001
?	18.06	2.40	0.78	0.034
Palmitic acid [b]	18.41	1.98	1.39	0.020
?	18.9	1.56	0.17	< 0.0001
Myo-inositol [b]	19.17	1.59	0.86	0.004
?	19.98	1.84	2.42	0.008
Stearic acid [b]	20.20	3.36	0.76	< 0.0001
Cholesterol [c]	29.96	2.09	1.15	0.024

[a] Retention time in the analysis by GC-MS.

[b] Identified using commercially available standards.

[c] Identified by comparing with the MS library.

[d] Not identified.

Table S7. A list of differential metabolites identified in kidney.

Metabolites	t_R (min)	Group ii (treated with CU (1:2)) *vs*. Group i (treated with saline)		
		VIP score	Fold change	t-test (*p*)
L-Lactic acid [c]	6.17 [a]	21.32	1.54	< 0.0001
Oxalic acid [c]	7.84 [a]	1.28	1.15	0.009
L-Leucine [c]	10.91 [a]	1.11	1.52	< 0.0001
? [e]	11.04 [a]	2.19	1.19	0.029
L-Proline [c]	11.46 [a]	1.08	1.55	< 0.0001
Glycine [c]	11.76 [a]	1.47	2.01	0.025
L-Serine [c]	13.14 [a]	22.20	1.49	< 0.0001
Aspartic acid [c]	16.98 [a]	22.37	1.41	< 0.0001
Cysteine [c]	17.74 [a]	21.97	1.38	< 0.0001
L-Glutamic acid [c]	19.23 [a]	1.09	1.28	0.001
Taurine [c]	20.24 [a]	1.08	0.67	0.003
Asparagine [c]	20.37 [a]	15.86	1.63	0.007
?	20.87 [a]	1.23	1.56	< 0.0001
Phosphoric acid [d]	22.57 [a]	1.14	0.80	0.002
Hypoxanthine [d]	23.07 [a]	1.11	1.42	< 0.0001
D-Glucose [c]	25.49 [a]	1.44	1.53	0.034
Cholesterol [d]	37.21 [a]	1.09	1.15	0.037
?	1.08 [b]	1.42	1.69	0.002
Nicotinamide [c]	1.13 [b]	5.44	1.27	0.030
Allopurinol [d]	1.21 [b]	6.81	1.30	0.050

Inosine [c]	1.25 [b]	1.76	1.28	0.020
L-Leucine [c]	1.32 [b]	5.31	1.40	0.001
?	1.48 [b]	3.13	1.25	0.020
?	1.48 [b]	3.09	1.89	< 0.0001
Tryptophan [c]	1.67 [b]	1.89	1.40	0.001
?	1.8 [b]	2.43	2.16	< 0.0001
?	2.34 [b]	1.61	1.63	0.001
LysoPC(0:0/20:4) [d]	4.84 [b]	3.64	1.32	0.030
LysoPC(16:0) [d]	5.18 [b]	4.66	1.50	0.020
LysoPC(18:1) [d]	5.77 [b]	7.13	1.46	0.010
?	6.22 [b]	1.39	5.46	0.004
?	6.29 [b]	3.16	1.65	0.010
LysoPC(0:0/18:0) [d]	6.72 [b]	1.03	6.35	< 0.0001
?	6.83 [b]	1.28	2.28	0.02

[a] Retention time in the analysis by GC-MS.

[b] Retention time in the analysis by LC-MS/MS.

[c] Identified using commercially available standards.

[d] Identified by comparing with the MS library.

[e] Not identified.

Table S8. A list of differential metabolites identified in liver.

Metabolite	t_R (min)	Group ii (treated with CU (1:2)) *vs*. Group i (treated with saline)		
		VIP score	Fold change	t-test (*p*)
? [e]	7.43 [a]	1.61	0.55	0.014
Phosphoric acid [d]	11.01 [a]	8.66	0.90	0.000
L-Proline [c]	11.45 [a]	1.38	0.70	0.001
Aspartic acid [c]	16.97 [a]	1.61	0.72	0.000
Threonic acid [c]	18.12 [a]	4.1	0.53	0.021
Arabinose [d]	20.85 [a]	2.41	1.54	0.020
Glutamine [c]	22.53 [a]	2.62	0.75	0.005
Ornithine [c]	23.49 [a]	1.17	0.77	0.001
Gluconic acid [d]	26.61 [a]	4.74	0.54	0.004
Palmitic acid [c]	27.25 [a]	1.08	0.91	0.039
Stearic acid [c]	29.67 [a]	2.29	0.87	0.007
Inosine [c]	32.97 [a]	5.22	2.81	0.015
Adenosine [c]	36.63 [a]	2.79	0.39	0.000
Cholesterol [d]	37.19 [a]	2.69	0.92	0.038
Taurocholic acid [d]	2.26 [b]	1.69	5.08	0.020
LysoPC(0:0/18:0) [d]	6.76 [b]	2.52	3.37	0.040

[a] Retention time in the analysis by GC-MS.

[b] Retention time in the analysis by LC-MS/MS.

[c] Identified using commercially available standards.

[d] Identified by comparing with the MS library.

[e] Not identified.

Table S9. Summary of the pathway analysis results.

Pathway name	Total compounds	Hits	-log(*p*)	Impact	Sample
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	16.19	1.00	Serum
Phenylalanine metabolism	12	2	16.19	0.36	
Aminoacyl-tRNA biosynthesis	48	10	14.81	0.17	
Glycine, serine and threonine metabolism	34	3	14.49	0.23	
Taurine and hypotaurine metabolism	8	1	13.59	0.43	
Arginine and proline metabolism	38	4	13.49	0.27	
Arginine biosynthesis	14	3	10.25	0.18	
D-Glutamine and D-glutamate metabolism	6	1	8.50	0.50	
Pyruvate metabolism	22	2	6.49	0.29	
Cysteine and methionine metabolism	33	2	6.39	0.12	Kidney
Arginine and proline metabolism	38	2	6.17	0.16	
Valine, leucine and isoleucine degradation	40	1	5.55	0.00	
Valine, leucine and isoleucine biosynthesis	8	1	5.55	0.00	
Purine metabolism	66	1	5.51	0.02	
Sphingolipid metabolism	21	1	5.13	0.00	
Pantothenate and CoA biosynthesis	19	2	4.87	0.00	
Pyruvate metabolism	22	1	4.70	0.08	
Taurine and hypotaurine metabolism	8	1	4.58	0.00	
Alanine, aspartate and glutamate metabolism	28	3	3.97	0.42	
Arginine biosynthesis	14	2	3.97	0.12	

Tryptophan metabolism	41	1	3.26	0.14	
D-Glutamine and D-glutamate metabolism	6	1	3.61	0.50	
Aminoacyl-tRNA biosynthesis	48	9	2.39	0.17	
Glycine, serine and threonine metabolism	34	3	1.77	0.48	
Arginine and proline metabolism	38	2	3.36	0.19	Liver
Aminoacyl-tRNA biosynthesis	48	3	3.05	0.00	
Glutathione metabolism	28	1	2.88	0.00	
Arginine biosynthesis	14	3	2.80	0.06	
Alanine, aspartate and glutamate metabolism	28	2	2.73	0.34	
Purine metabolism	66	3	2.62	0.00	
Pentose phosphate pathway	22	1	2.51	0.05	
Glycerophospholipid metabolism	36	1	1.36	0.02	

S5. Biochemical assays associated with oxidative stress

S5.1. SOD assay

The assay was conducted according to the manufacturer's protocol. In brief, tissue samples (10 mg) were homogenized in ice-cold 0.1 M Tris·HCl buffer (pH 7.4) containing 0.5% Triton X-100, 5 mM β-mercaptoethanol, and 0.1 mg mL^{-1} phenylmethylsulfonylfluoride. After centrifugation at 14,680 *g* for 10 min, the supernatant expressing total SOD activity from mitochondrial and cytosolic enzymes was transferred to a clean tube. All materials and reagents were equilibrated to room temperature and gently agitated just prior to use unless specified. Each reaction well in a 96-well plate was set-up according to Table S10. After mixing of samples and reagent solutions and incubation at 37 °C for 20 min, absorbance at 450 nm was measured. SOD activity was calculated using the following equation:

$$SOD\ activity\ (\%) = \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \times 100$$

Table S10. SOD assay conditions.

Component	Sample (μL)	Blank 1 (μL)	Blank 2 (μL)	Blank 3 (μL)
Sample solution	20	0	20	0
H_2O	0	20	0	20
WST [a] working solution	200	200	200	200
Enzyme working solution	20	20	0	0
Dilution buffer	0	0	20	20

[a] A tetrazolium salt that produces a water-soluble formazan dye upon reduction with superoxide anion.

S5.2. CAT assay

The manufacturer's protocol was applied without modifications. For a standard calibration curve, a series of H_2O_2 standard solutions (0, 20, 40, 60, 80, and 100 µM using 1 mM H_2O_2) were prepared in assay buffer, and 90 µL of each solution was removed and mixed with 10 µL of stop solution. For tissue samples, 100 mg of liver or 50 mg of kidney were homogenized with 200 µL of assay buffer and centrifuged at 14,680 *g* for 10 min. The collected supernatant was stored on ice until use. The cleared lysates of liver (10 µL) and kidney (20 µL) were added to two series of wells and adjusted to a final volume to 78 µL using the assay buffer. For the high control (HC) sample series, stop solution (10 µL) was added and incubated at 25 ºC for 5 min to completely inhibit the CAT activity. Both the regular and HC samples were mixed with 12 µL of 1 mM H_2O_2. After incubation at 25 ºC for 30 min, only the regular sample was mixed with 10 µL of stop solution. Finally, 50 µL of developer mix solution, horseradish peroxidase (HRP), and peroxidase substrate (OxiRed probe) was added to all the wells and incubated at 25 ºC for 10 min with protection from light. Absorbance was measured at 570 nm. Mean absorbance of the blank (containing no H_2O_2) was subtracted from the absorbance of all standards and samples. The corrected readings of the standard solutions were plotted against the H_2O_2 concentrations to establish a standard calibration curve using Prism Ver. 7.0. For each sample, ΔAbs (= A_{HC}- A_{sample}; A_{HC} and A_{sample} are the readings of the HC and regular samples, respectively) was obtained and applied to the H_2O_2 calibration curve to determine the concentration of H_2O_2 (µmol) decomposed by CAT during the 30 min reaction. CAT activity (µmol $min^{-1}mL^{-1}$) in the tested sample was calculated using the following equation:

$$CAT\ activity\ (\mu mol\ min^{-1}\ mL^{-1}) = \frac{B}{30 \times V}(\times D)$$

where B is the amount of H_2O_2 in a sample well (nmol), 30 is the CAT reaction time (min), V is the pre-original sample volume added into the reaction well (mL), and D is the sample dilution factor (D = 1 in this study).

S5.3. MDA assay

The manufacturer's protocol was followed without any modifications. A stock solution (4.17 M) of malondialdehyde (MDA) was serially diluted with water to prepare 2 mM MDA standard solution and was used to prepare a series of MDA standard solutions (0, 20, 40, 60, 80, and 100 µM). For samples, 10 mg of tissues were homogenized with 303 µL of the MDA lysis buffer and centrifuged at 14,680 *g* for 10 min. Each vial containing 200 µL of standard and 200 µL of sample was mixed with 600 µL of thiobarbituric acid (TBA) solution, resulting in the generation of MDA-TBA adduct. After incubation at 95 ℃ for 1 h, the vial was cooled on ice-bath for 10 min. Samples became turbid, and they were filtered through a 0.2-µm syringe filter (Whatman, Piscataway, NJ, USA) for clarity before absorbance reading. A portion of the reacted solution (200 µL) was transferred to a well, and its absorbance was measured at 532 nm.

Mean absorbance of the blank (containing no MDA) was subtracted from the absorbance of all standards and samples. The corrected readings of the standard solutions versus the MDA levels were plotted to construct a standard calibration curve of MDA. The sample absorbance was applied to the standard curve to quantify the MDA levels in each sample well. The MDA concentration in the tissue sample was calculated using the following equation:

$$MDA\ concentration\ (nmol\ mg^{-1}) = \frac{A}{\mu g} \times 4\ (\times\ D)$$

where A is the amount (nmol) of MDA in a sample calculated from the standard curve, mg is the original amount of tissue used, 4 is the correction factor for using 200 µL of the 800 µL reaction mixture, and D is the sample dilution factor (D = 1 in this study).

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S5.4. GSH/GSSG assay

The manufacturer's protocol was followed without modifications. A series of glutathione (GSH) standard solutions were prepared using assay buffer at the final concentrations of 0, 0.1563, 0.3125, 0.625, 1.25, 2.5, 5.0, and 10 μM. For the glutathione disulfide (GSSH) standard solutions, the final concentrations were 0, 0.0781, 0.1563, 0.3125, 0.625, 1.25, 2.5, and 5.0 μM. A portion (20 mg) of liver or kidney was homogenized in 400 μL of ice-cold PBS containing 0.5% NP-40 and centrifuged at 14,680 *g* for 10 min. The supernatant was collected and kept on ice until use. A 96-well plate was split into two panels: panel A for GSH measurement and panel B for GSSG measurement. Panel A had two replicates of a series of GSH standard solution, followed by columns of test samples in eight replicates. Likewise, the layout of panel B consisted of columns of GSSG standard solutions in duplicates, followed by columns of test samples. The wells were filled with 50 μL of GSH standard, GSSH standard, or sample solution. GSH assay mixture (GAM) was prepared by diluting 50 μL of 100× stock solution of thiol green with 5 mL of assay buffer for GSH detection, while total GSH assay mixture (TGAM) was prepared by diluting 100 μL of 25× stock solution of GSSG probe with 2.5 mL of GAM for detection of GSH and GSSG. Finally, 50 μL of GAM or TGAM were added to each well to make 100 μL in total, and incubated at room temperature for 60 min. Fluorescence was monitored at Ex/Em = 490 nm/520 nm.

Mean absorbance of the blank (containing no GSH or GSSG standard) was subtracted from the absorbance in all standard and sample readings. The corrected values were plotted against log-transformed concentrations of GSH and total GSH+GSSG to construct standard calibration curves. Concentrations of GSH and total GSH+GSSG were calculated using the calibration curves, and the GSSG concentration could be estimated using the following equation:

$$GSSG\ (\mu M) = \frac{Total\ glutathione - GSH}{2}$$

The results of SOD activity, CAT activity, GSH/GSSG, MDA assays are summarized in Table S11.

Table S11. Results (mean ± SD) of the oxidative stress-associated assays shown in Figure 5.

Mouse group	Superoxide dismutase (SOD) activity (%)		Catalase (CAT) activity (μmol min^{-1} mL^{-1})		Ratio of glutathione to glutathione disulfide (GSH/GSSG)		Malondialdehyde (MDA) level (nmol mg^{-1})	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Group i	99.4 (± 0.5)	99.2 (± 0.3)	4.41 (± 1.28)	0.19 (± 0.10)	2.08 (± 0.44)	7.25 (± 0.62)	0.288 (± 0.100)	0.025 (± 0.009)
Group ii	99.9 (± 0.6) [a]	95.9 (± 2.1) [d]	4.65 (± 0.79)	0.11 (± 0.07) [f]	0.35 (± 0.59) [h]	7.01 (± 1.14)	0.386 (± 0.166)	0.049 (± 0.021) [k]
Group iii	98.5 (± 0.5) [b]	98.0 (± 0.7)	3.99 (± 0.74)	0.21 (± 0.08)	2.78 (± 0.16)	8.13 (± 1.06)	0.332 (± 0.058)	0.035 (± 0.012)
Group iv	99.0 (± 0.6)	97.8 (± 2.2)	3.67 (± 1.12)	0.09 (± 0.86) [g]	1.54 (± 1.62)	8.77 (± 1.80) [i]	0.341 (± 0.120)	0.032 (±0.013)
Group v	99.9 (± 0.3) [c]	97.0 (± 1.3) [e]	3.60 (± 1.39)	0.11 (± 0.04)	2.63 (± 0.79)	8.96 (± 1.38) [j]	0.322 (± 0.054)	0.014 (±0.001)

[a] $p = 0.0252$ between group i and group ii.

[b] $p = 0.0025$ between group i and group iii.

[c] $p = 0.0444$ between group i and group v.

[d] $p = 0.0008$ between group i and group ii.

[e] $p = 0.0234$ between group i and group v.

[f] $p = 0.0375$ between group i and group ii.

[g] $p = 0.0353$ between group i and group iv.

[h] $p = 0.0018$ between group i and group ii.

[i] $p = 0.0177$ between group i and group iv.

[j] $p = 0.0107$ between group i and group v.

[k] $p = 0.0023$ between group i and group ii.

S6. Ammonia assay and calculation of the theoretical amount of total ammonia administered to mice

The assay was performed according to the manufacturer's protocol. Ammonia assay reagent containing α-ketoglutaric acid and NADPH was prepared and 1.0 mL of it was added to each cuvette. Reagent blank and standard cuvettes contained 100 µL of water and ammonia standard solution (10 µg mL^{-1}), respectively, while sample cuvette contained 100 µL of test solution (e.g., CU and mouse serum). All the contents in a cuvette were mixed and incubated at room temperature for 5 min, and absorbance ($A_{Initial}$) was measured at 340 nm. Next, 10 µL of L-glutamate dehydrogenase (GDH) solution was added and incubated for another 5 min. The absorbance was measured again at 340 nm (A_{Final}). The calculation procedures are as follows:

$$\Delta A_{340} = A_{Initial} - A_{Final}$$

$$\Delta(\Delta A_{340})\ for\ test\ or\ standard = \Delta A_{340}(test\ or\ standard) - \Delta A_{340}(blank)$$

$$mg\ of\ NH_3\ of\ original\ sample\ (1\ mL) = \frac{\Delta(\Delta\ \text{A340})\ \times TV\ \times MW\ of\ ammonia\ \times F}{\varepsilon\ \times d\ \times SV\ \times 1000} = \frac{\Delta(\Delta\ \text{A340})\ \times TV \times F}{SV} \times 0.00273$$

where TV is total assay volume (mL), SV is sample volume (mL), MW of ammonia is 17 g $mole^{-1}$, F is dilution factor from sample preparation, ε is millimolar extinction coefficient (mM^{-1} cm^{-1}) for NADPH at 340 nm, and d is pathlength (cm).

The concentration of ammonia in CU that had been freshly prepared using the heating method was found to range in 42.8–244.4 µg mL^{-1}. As displayed in Table S5, group ii mice received CU at a dose of 1.5 g kg^{-1} by administering 70.4 µL of two-fold diluted CU (CU:water, 1:1 v/v). Thus, the volume of CU directly administered to mice is $70.4\ \mu L \times \frac{1}{2}$. By applying the average weight of mice (28 g), the theoretical amount of total ammonia administered to mice can be calculated as follows:

for 42.8 µg mL^{-1} of ammonia in CU (1:2),

$$(42.8\ \mu g\ mL^{-1} \times 10^{-3}\ mg\ \mu g^{-1}) \times \left(70.4\ \mu L \times \frac{1}{2} \times 10^{-3}\ mL\ \mu L^{-1}\right) \div (28\ g \times 10^{-3}\ kg\ g^{-1}) = 0.054\ mg\ kg^{-1}$$

and

for 244.4 µg mL^{-1} of ammonia in CU (1:2),

$$(244.4\ \mu g\ mL^{-1} \times 10^{-3}\ mg\ \mu g^{-1}) \times \left(70.4\ \mu L \times \frac{1}{2} \times 10^{-3}\ mL\ \mu L^{-1}\right) \div (28\ g \times 10^{-3}\ kg\ g^{-1}) = 0.307\ mg\ kg^{-1}$$

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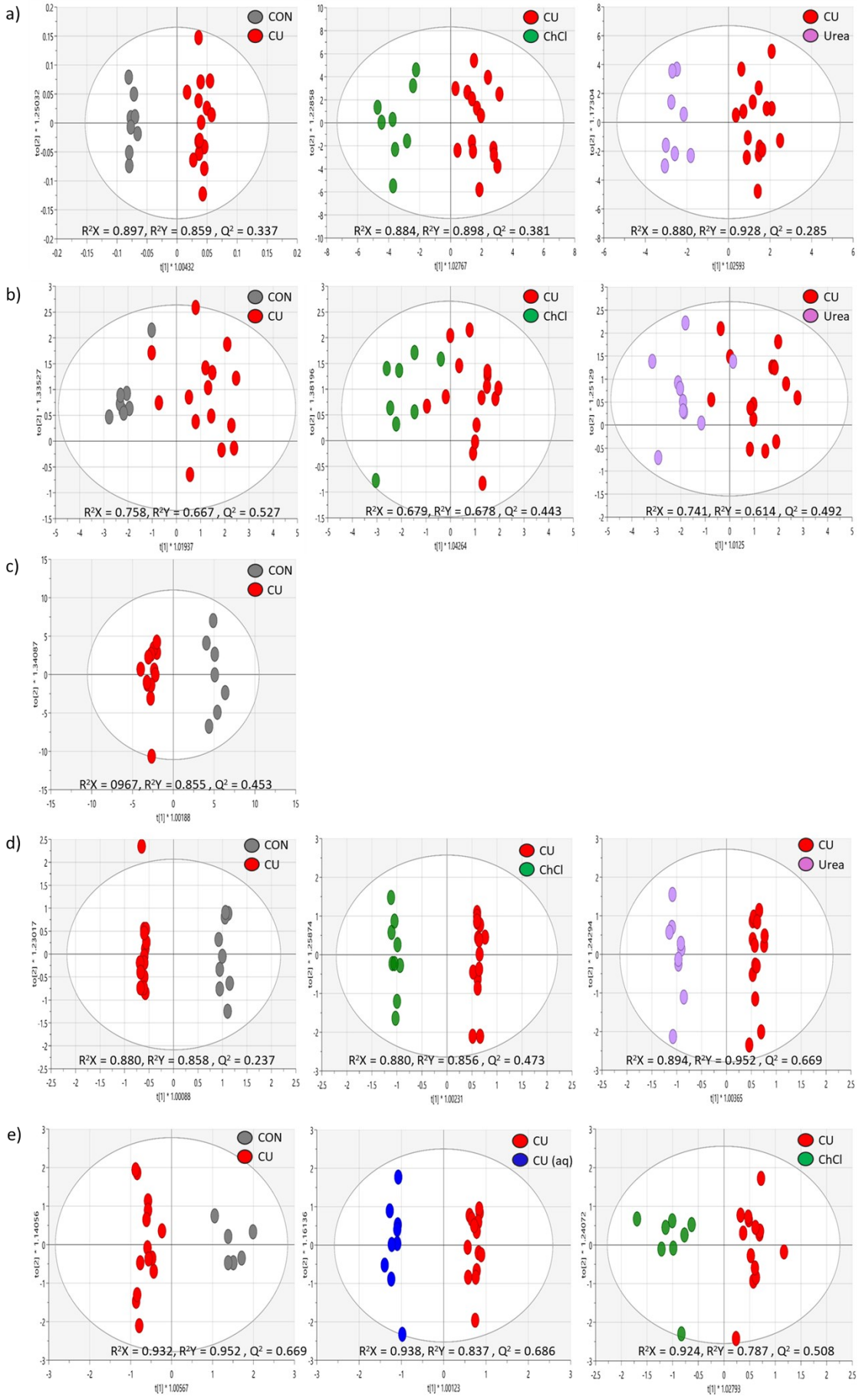
S7. OPLS-DA results

Fig. S1. OPLS-DA score plots for pair-wise comparisons of the CU (1:2)-treated group with the other groups. (a) Serum, (b, d) kidney, and (c, e) liver samples were analyzed by GC-MS (a, b, c) and LC-MS/MS (d, e). Group identification: CON, group i (treated with saline); CU, group ii (treated with CU (1:2)); CU (aq), group iii (treated with CU (1:2) (aq)); ChCl, group iv (treated with ChCl); Urea, group v (treated with urea). Detailed information on the doses is provided in Table S5.


a)
CON
CU
R²X = 0.897, R²Y = 0.859 , Q² = 0.337
CU
ChCl
R²X = 0.884, R²Y = 0.898 , Q² = 0.381
CU
Urea
R²X = 0.880, R²Y = 0.928 , Q² = 0.285
b)
CON
CU
R²X = 0.758, R²Y = 0.667 , Q² = 0.527
CU
ChCl
R²X = 0.679, R²Y = 0.678 , Q² = 0.443
CU
Urea
R²X = 0.741, R²Y = 0.614 , Q² = 0.492
c)
CON
CU
R²X = 0967, R²Y = 0.855 , Q² = 0.453
d)
CON
CU
R²X = 0.880, R²Y = 0.858 , Q² = 0.237
CU
ChCl
R²X = 0.880, R²Y = 0.856 , Q² = 0.473
CU
Urea
R²X = 0.894, R²Y = 0.952 , Q² = 0.669
e)
CON
CU
R²X = 0.932, R²Y = 0.952 , Q² = 0.669
CU
CU (aq)
R²X = 0.938, R²Y = 0.837 , Q² = 0.686
CU
ChCl
R²X = 0.924, R²Y = 0.787 , Q² = 0.508