Experimental methods

1 Materials

CK was purchased from Shanghai Yuanye Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Clark Bioscience (Claymont, DE, USA). Penicillin and streptomycin were purchased from Biosharp (Hefei, China). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). An Easy Protein Quantitative kit was purchased from Beyotime (Beijing, China).

2 Cells and treatments

HT22 mouse hippocampal neurons were purchased from Shanghai EK-Bioscience Biotechnology Co., Ltd (Shanghai, China). Cells were cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humid atmosphere with 5% CO_2. CK was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in cell culture medium as needed. Cells were divided into five groups: control group, model group [(400 μM hydrogen peroxide (H_2O_2)], and three drug-treated groups, CK1-treated (4 μM CK + 400 μM H_2O_2), CK2-treated (6 μM CK + 400 μM H_2O_2), and CK3-treated (8 μM CK + 400 μM H_2O_2) groups.

3 MTT assay of cell viability

HT22 cells were seeded at a density of 5×10^4 cells/well in 96-well plates for 24 h, treated with CK (4, 6, or 8 μM) for 24 h, and incubated with H_2O_2 for 4 h. Then, 20 μL MTT was added to each well, and cells were incubated for 4 h. Next, 150 μL DMSO was added to each well for 5 min, and the absorbance of the resulting solution in each well was measured three times at 490 nm.

4 Sample preparation

HT22 cells were added to culture flasks at a concentration of 1×10^8 cells per flask. Six parallel flasks were assigned to each group. Following treatment with CK for 24 h, cells were incubated with 400 μM H_2O_2 for 4 h. To determine the absorbance of the culture medium, samples were centrifuged for 10 min at 135 × *g* and 4 ℃. Then, cells were rinsed with phosphate-buffered saline (PBS) eight times and digested with 0.25% trypsin. Pelleted cells were gently resuspended in PBS and after combining both groups, cells were centrifuged twice under the same conditions. Then, the supernatants were discarded, and cells were frozen in liquid nitrogen and

stored overnight at −80 ℃. The cells and culture medium were collected and freeze-thawed at 4 ℃ five times. Then, a methanol-water (1:2, v/v) solution was added. After vortexing, cells were lysed by sonication in an ice bath (5 s on and 9 s off for 20 min). To harvest the cellular content, suspensions were centrifuged for 10 min at 15,871 × *g* and 4 ℃, and the supernatants were freeze-dried for cell metabolomics analysis.

5 ^{1}H-NMR analysis and data acquisition

The freeze-dried cellular powders were dissolved in 600 μL 0.1 M phosphate buffer (containing 0.005% TSP in 10% deuterated water, pH 7.4), and the mixtures were centrifuged for 10 min at 15,871 × *g* and 4 ℃. The supernatants were placed in a 5 mm ^{1}H-NMR tube and all samples were analyzed using a BrukerAve Ⅲ 600 spectrometer at 25 ℃ (frequency, 600 MHz) using nuclear Overhauser effect spectroscopy (NOESY) with a relaxation delay of 320 ms and spectral width of 12,019.12 Hz. The scanning frequency was 64 times using TSP as the internal standard.

6 Data processing and analysis

Raw data were processed using MestReNova software (version 7.1.2, Mestrelab Research Company, Spain). Both phase adjustment and baseline correction were performed. The acquired H-NMR data were analyzed by pattern recognition. TSP (δ 0.00) was considered the position of the chemical shift reference peak. The 5.2–4.7 ppm peak was removed to exclude the chemical shift of water. Integral data were normalized to eliminate systematic differences between sample concentrations. Principal component analysis (PCA) was performed on the integration value dataset using SIMCA-P+ (version 12.0, Sweden Umetrics Company).

P-values < 0.05 were considered significant. Metabolites with variable importance (VIP) greater than 1 were considered potential biomarkers, and potential biomarkers were identified by searching the human metabolome database (http://www.hmdb.ca/). The MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) online analysis platform was used to identify metabolic pathways related to potential biomarkers.

7 Western blotting

Proteins in total cell lysates were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk for 2 h at 37 °C, washed

with Tris-buffered saline + Tween-20 buffer, and incubated with GSK-3β (Bioss, China), AKT (Bioss), PI3K (Bioss), and β-actin (Bioss) antibodies for 24 h at 4 °C. After incubation with HRP-conjugated secondary antibodies (Bioss) for 1 h at 37 °C, protein bands were visualized using a chemiluminescence imaging system (APLEGEN INC, USA).

8 ATP content measurement

ATP (Beyotime Biotechnology, China) detection solution was diluted to generate standard solutions with different ATP concentrations and added to each well. These concentrations were measured and used to generate a standard curve. Cells were lysed and supernatants were collected for subsequent measurements. Finally, ATP contents were calculated using the standard curve.

9 Statistics

Data are expressed as mean ± standard error of the mean. One-way analysis of variance followed by Student's t-test was performed with GraphPad Prism 6.0 (GraphPad Software, USA). $P < 0.05$ and $P < 0.01$ were considered statistically significant.