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cInvestigating the protective effects of ginsenoside CK against oxidative stress-induced neuronal damage by 1H-NMR-based metabolomics

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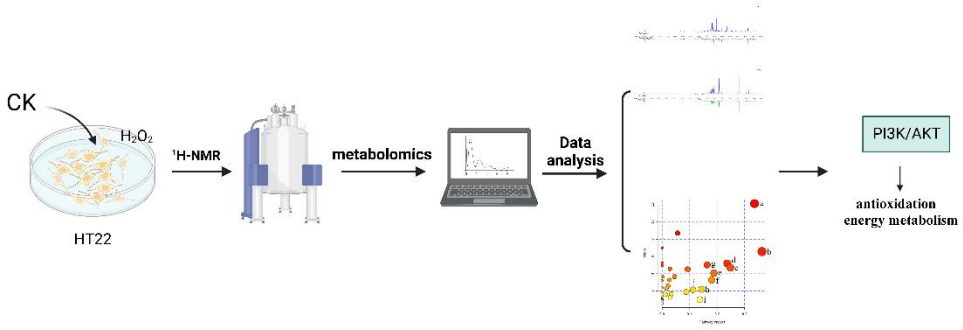

Highlights

A preliminary exploration of the endogenous metabolites involved in the effects of CK in damaged HT22 cells was conducted by ^{1}H-NMR.

CK can affect taurine, glycine, glutamate and glutathione metabolism by metabolomic analysis.

CK regulates ATP content in oxidatively damaged HT22 cells by upregulating the expression of PI3K/AKT signaling pathway.

In brief

CK can affects energy metabolism pathways and against neuronal oxidative stress by PI3K/AKT signaling pathway.

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Abstract

Oxidative stress is an important pathogenic mechanism in degenerative diseases such as Alzheimer's disease (AD). Although ginsenoside compound K (CK) is protective against neuronal oxidative damage, the underlying mechanism remains to be understood. In this study, the protective effects of ginsenoside CK against hydrogen peroxide-induced oxidative stress damage in HT22 cells were investigated by ^{1}H nuclear magnetic resonance (^{1}H-NMR)-based metabolomics. The optimal CK concentration for reducing oxidative stress damage in nerves was determined using the MTT. CK (8 μM) significantly increased the HT22 cell survival rate after the model was established. Cell lysates were subjected to ^{1}H-NMR metabolomics, western blotting, and ATP assays for verification. Metabolic perturbation occurred in HT22 cells in the model group but not in the control group. Twenty biomarkers were identified in this study and used to analyze metabolic pathways. CK reversed metabolic changes in HT22 cells by altering taurine, glutamate, glycine, and glutathione metabolism. Subsequently, CK increased ATP content and expression of components of the PI3K/AKT signaling pathway in HT22 cells. These findings demonstrated CK can prevent oxidative stress damage and protect nerves by regulating energy metabolism pathways, such as taurine and glutamate metabolism and other amino acid metabolism pathways, providing a rationale for CK in AD.

Keywords: Oxidative stress; ginsenoside CK; metabolomics; ATP

Alzheimer's disease (AD) is a life-threatening neurodegenerative disorder. The primary clinical manifestations of AD are cognitive impairment, intellectual decline, and personality change [1]. With aging of human society, the number of AD patients continues to increase. AD is caused by genetic, environmental, and lifestyle factors that impact neuronal cell degradation over time and seriously affect the quality of life of elderly individuals. Intensive research on the pathogenesis of AD is ongoing [2].

One of the main causes of AD is oxidative stress, which leads to neuronal injury and death. Oxidative stress is caused by an imbalance in the production and elimination of oxygen free radicals, resulting in the accumulation of oxygen active substances in the body and a subsequent stress response [3]. High levels of reactive oxygen species (ROS) can cause oxidation of DNA, proteins, lipids, and other biological macromolecules, affection the function and structure of biological

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molecules; it can also alter signal transduction, resulting in abnormal cell function and apoptosis. Therefore, inhibiting oxidative stress may be a feasible approach in the treatment of AD [4].

The history of ginseng (*Panax ginseng* C. A. Mey.) is described in "Shen Nong's Materia Medica". Ginseng exerts traditional effects, such as nourishing vitality, nourishing the spleen and lungs, delaying nourishment, and rejuvenating the nerves; its individual components and metabolic components also show biological activity [5]. Ginsenoside compound K [20-O-β-D-glucopyranosyl-20(S)-protopanaxadiaol (CK)], one of the primary active metabolites of protopanaxadiol-type ginsenosides, is produced by intestinal flora from ginsengdiol saponins; moreover, CK has biological properties, such as antiaging, neuroprotective, antioxidative, and anti-inflammatory [6]. In our previous study [7], we confirmed that CK has neuroprotective properties; however, there have been no analyses of antioxidative and neuroprotective effects of CK using ^{1}H nuclear magnetic resonance (^{1}H-NMR)-based metabolomics.

Recently, metabolomic techniques, an important part of “systems biology” approaches, have been widely used to discover novel biomarkers [8; 9]. ^{1}H-NMR-based metabolomics have been widely used to examine overall metabolic changes in organisms, allowing the efficacy of traditional Chinese medicine formulations, including their pharmacological and toxicological mechanisms, to be evaluated [10]. Cell metabolomics research has rapidly and intuitively described the metabolism of specific cell types with little interference, revealing the overall performance of microscopic physiological functions [11].

Neuronal damage caused by oxidative stress can lead to neurodegenerative diseases such as AD. Excessive production of ROS triggers intracellular oxidative stress. Hydrogen peroxide (H_2O_2) is a reactive oxygen species, an important inducer of oxidative stress, and is used in the study of neurodegenerative diseases such as AD caused by oxidative stress [12]. Therefore, we treated HT22 cells with H_2O_2 to establish an intracellular model of AD. MTT assay results showed that 8 μM CK significantly increased the survival rate of HT22 cells (Figure 1). Thus, 8 μM CK was used for metabolomics analysis.

Second, the potential protective effects of CK against H_2O_2-induced changes in endogenous metabolites of HT22 cells were investigated using ^{1}H-NMR-based

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metabolomics. Specifically, differential metabolites in each treatment group were screened and identified, and the primary metabolic pathways were analyzed.

Analysis of the ^{1}H-NMR data combined with a comparison of previous studies allowed assignment of signals of the main metabolite components in the three groups of cells (Figure 2, Table S1). The peak value of the ^{1}H-NMR spectra was normalized using MestReNova software, and the normalized peak data were imported into SIMCA-P software for PLS-DA analyses.

The PLS-DA method removes interference factors that are not related to sample classification and maximizes the difference between groups. The results showed differences in the cell metabolite composition between the control and model groups (Figure 3A). The values of parameters R^2Y and Q^2Y were 0.815 and 0.986, respectively; this showed that a PLS-DA model with good fit and predictability had been established (Figure 3B). The CK group cluster was distinct from the model group cluster (Figure 3C), indicating that the CK group is different from the model group.

The loading plot (Figure 3D) shows that the metabolites farther away from the origin could be regarded as candidate biomarkers. The metabolites with VIP values (VIP1 and VIP2) > 1 and $P < 0.05$ were highlighted as potential biomarkers. A total of 20 metabolites were highlighted as potential biomarkers that inhibit oxidative stress injury, and detailed information about these biomarkers and trends in changes in these biomarkers between groups are shown in Table 1.

These biomarkers were imported into the MetaboAnalyst 3.0 online analysis tool to perform enrichment and pathway analyses (metabolic pathways with an influence value > 0.1). The results suggest CK regulates taurine and hypotaurine metabolism, pyruvate metabolism, alanine aspartate and glutamate metabolism, and glycine serine and threonine metabolism (Figure 4A and B) to reduce oxidative stress damage.

Taurine, a well-known antioxidant, has potential antiapoptotic properties and exerts important regulatory effects on brain function; it has neuroprotective effects and improves cognitive function [13]. Taurine is a key protein transformation node involved in amino acid and energy metabolism; it can limit peroxidation, regulate glutamate metabolism, inhibit nerve cell apoptosis, and protect nerve cell function. Increasing evidence suggests that taurine-induced neuroprotection is mediated by the antagonism of glutamate-induced excitotoxicity [14]. Taurine content was greatly

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increased in the model groups compared with the control group, which may be a compensatory response to antioxidant damage [15; 16]. Taurine content was decreased following treatment with CK, indicating that CK reduced oxidative stress and eliminated the compensatory response.

Glycine is an amino acid with a simple structure that counteracts the production of ROS and attenuates H_2O_2-induced oxidative damage in neurons [17]. CK is also involved in glutathione metabolism and glycine is the main precursor of glutathione synthesis [18]. Glutathione is an endogenous antioxidant that plays a key role in the defense against oxidative stress in the brain and counteracts neurotoxicity due to peroxides such as H_2O_2 [19]. Impaired glutathione metabolism may lead to neurodegenerative diseases such as AD. Therefore, maintaining glutathione metabolism homeostasis plays a crucial role in neuronal protection [20]. Our results showed CK was involved in glycine metabolism and increased glycine content, and thus plays a role in regulating glutathione metabolism and reducing H_2O_2-induced oxidative damage in HT22 cells.

Glutamate, another essential precursor for glutathione synthesis, participates in glutathione metabolism [21]. Glutamate is also an important neurotransmitter in the cbrain. Intraneuronal homeostasis of glutamate is essential for neuronal energy metabolism and amino acid metabolism; glutamate affects synaptic transmission and brain function. Several studies have shown reduced glutamate levels in the brains of AD patients [22], which is consistent with the results of the present study suggesting CK may enhance ATP (Figure 5) levels in HT22 cells by regulating glutamate metabolism.

Some studies have indicated that phosphoinositide 3-kinase (PI3K)/ protein kinase B (PKA, also known as AKT) signaling is an important therapeutic target for the treatment of AD. Moreover, PI3K/AKT signaling is involved in the regulation of neuronal oxidative stress and energy metabolism in AD [23]. Therefore, in the third step, we detected the expression level of PI3K/AKT signaling pathway by Western Blotting. PI3K is a lipid kinase that generates phosphatidylinositol-3,4,5-trisphosphate, which in turn promotes the translocation of AKT to the plasma membrane. PI3K/AKT signaling influences neuronal plasticity, cell survival, proliferation, and apoptosis inhibition [24]. AKT, an important upstream regulator of GSK-3β, increases GSK-3β phosphorylation, thus inactivating GSK-3β. The antagonistic effects of GSK-3β

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activity influence central nervous system axon regeneration. Of note, inhibitors of GSK-3β have been postulated to exert neuroprotective effects [25]. The present results showed CK increased expression of PI3K/AKT signaling pathway components, and improved regulation of the expression of proteins involved in energy metabolism pathways.

We explored the effect of CK on endogenous metabolites in oxidatively damaged neurons *in vitro*. We found that CK also has neuroprotective effects *in vivo* [26]. Whether CK can cross the blood–brain barrier is unknown. Karpagam et al. showed CK was able to cross the blood–brain barrier by ADMET assay [27], but further investigation is needed to determine whether it can cross the blood–brain barrier *in vivo*.

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Conflicts of interest:

The authors declare no conflicts of interest.

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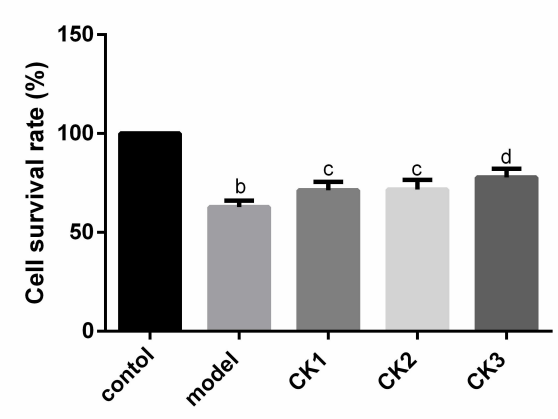

Figure 1. Effect of CK treatment on cell growth. Viability of cells treated with different concentrations of CK. Model: 400 μM H_2O_2, CK1: 4 μM CK + 400 μM H_2O_2, CK2: 6 μM CK + 400 μM H_2O_2, and CK3: 8 μM CK + 400 μM H_2O_2. $^{b}P < 0.01$ vs. control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs. model group. Values represent mean ± SD (n = 8).

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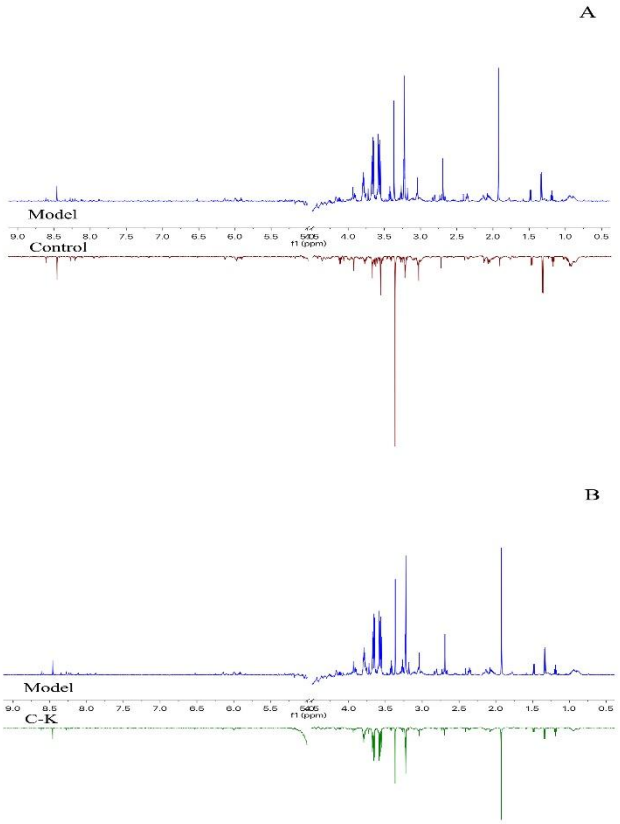

Figure 2. Typical ^{1}H NMR spectra of HT22 cells. (A) Control group and model group. (B) Model group and CK group.

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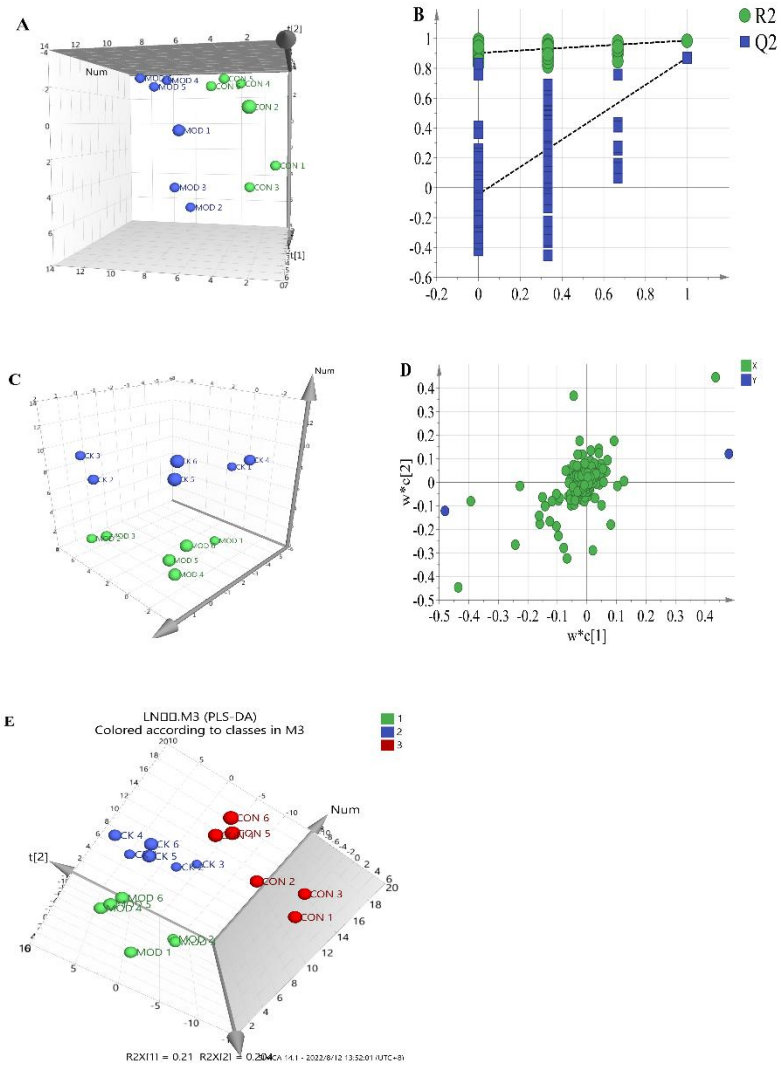

Figure 3. (A) PLS-DA score plot of the control and model groups. (B) Permutation test of the control and model groups. (C) PLS-DA score plot of the CK and model groups. (D) Loading plot. (E) PLS-DA score plot of the control, model, and CK groups.

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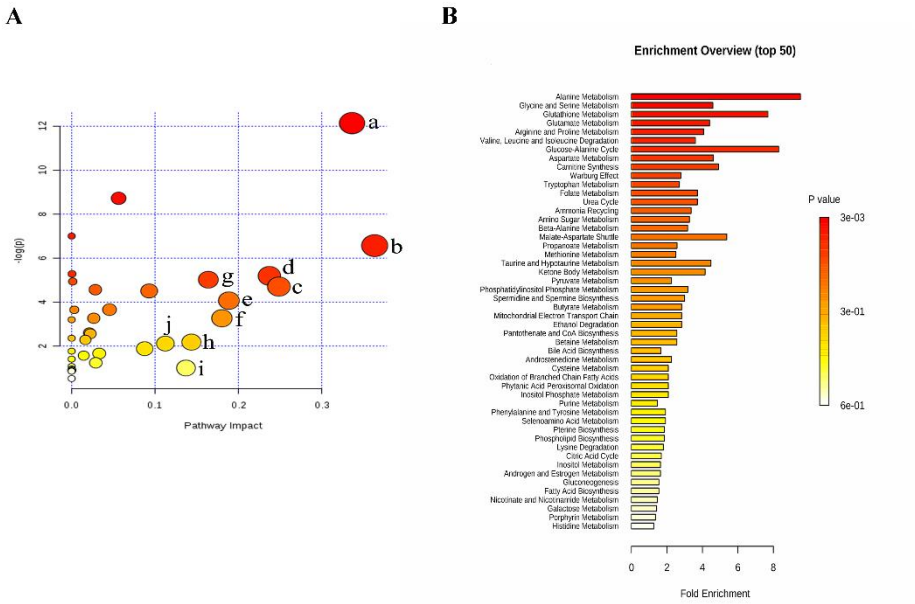

Figure 4. Altered metabolic pathways in H_2O_2-damaged cells visualized as (A) bubble plots and (B) an enrichment overview. a: Alanine, aspartate, and glutamate metabolism; b: taurine and hypotaurine metabolism; c: glutathione metabolism; d: pyruvate metabolism; e: glycine, serine, and threonine metabolism; f: pantothenate and CoA biosynthesis; g: methane metabolism; h: glyoxylate and dicarboxylate metabolism; i: inositol phosphate metabolism; and j: D-glutamine and D-glutamate metabolism.

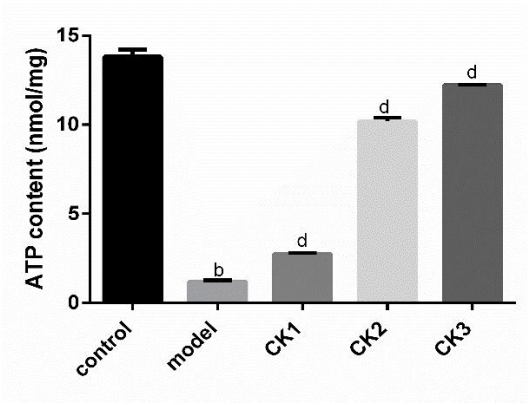

Figure 5. Effect of CK on intracellular ATP levels of HT22 cells. Model: 400 μM H_2O_2, CK1: 4 μM CK + 400 μM H_2O_2, CK2: 6 μM CK + 400 μM H_2O_2, and CK3: 8 μM CK + 400 μM H_2O_2. $^{b}P < 0.01$ vs. control group; $^{d}P < 0.01$ vs. model group. Values represent mean ± SD (n = 8).

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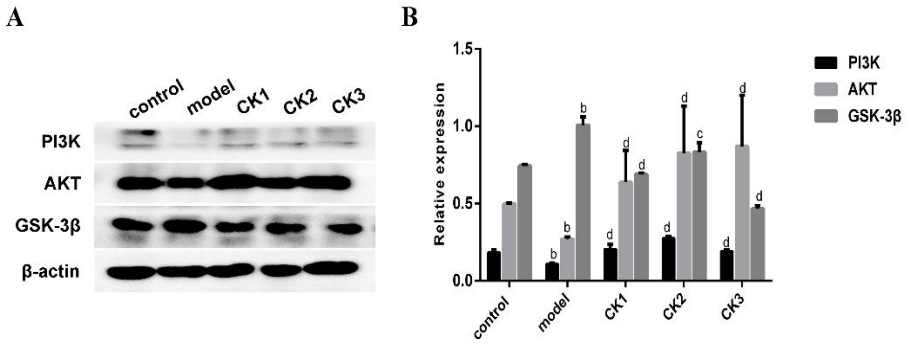

Figure 6. Western blotting analysis of expression of proteins related to energy metabolism. Model: 400 μM H_2O_2, CK1: 4 μM CK + 400 μM H_2O_2, CK2: 6 μM CK + 400 μM H_2O_2, and CK3: 8 μM CK + 400 μM H_2O_2 (A) and comparison of expression levels (B). $^{b}P < 0.01$ vs. control group; $^{c}P < 0.05$, $^{d}P < 0.01$ vs. model group. Values represent mean ± SD (n = 3).

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Table 1. Potential biomarkers of oxidative stress.

No.	ppm	MW	Metabolite	KEGG ID	Model/Control	CK/Model
1	3.238	146.20	Acetylcholine	C01996	↑b	↓d
2	3.278	125.15	Taurine	C00245	↑b	↓c
3	1.358	90.08	Lactate	C00186	↑b	↓c
4	3.598	75.07	Glycine	C00037	↓	↑[d]
5	3.838	89.09	Alanine	C00041	↑b	↓c
6	2.438	118.088	Succinic acid	C00042	↑b	↑
7	3.718	193.199	Phenylacetylglycine	C05598	↑b	↓c
8	1.958	60.05	Acetic acid	C00033	↓b	↑d
9	4.358	244.2	Uridine	C00299	↑a	↓
10	3.758	147.13	Glutamate	C00025	↓a	↑
11	0.958	131.18	Leucine	C00123	↑a	↓
12	2.678	149.21	Methionine	C00073	↑a	↓
13	3.958	131.133	Creatine	C00300	↑a	↓c
14	3.958	219.23	Pantothenic acid	C00864	↑a	↓c
15	3.198	162.20	O-Acetylcholine	C01996	↓a	↑d
16	8.478	45.01	Formate	C00058	↑a	↓
17	0.998	117.15	Valine	C00183	↑a	↓
18	2.038	175.14	N-Acetylaspartate	C01042	↓a	↑c
19	3.078	113.1	Creatinine	C00791	↓a	↓
20	3.558	180.16	Inositol	C00137	↓a	↑d

Notes: [a]$P < 0.05$, [b]$P < 0.01$, model group vs. control group; [c]$P < 0.05$, [d]$P < 0.01$, CK group vs. model group.

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Table S1. Metabolite components in cells in the control, model, and CK3 groups.

No.	Name	ppm
1	Bile acid	0.73(m)
2	Lipid	0.87(m)a, 1.28(m), 5.30(m)
3	Pantothenic acid	0.90(s), 3.96(s)
4	Leucine	0.95(dd), 1.70(m), 3.72(t), 1.78(m)
5	Valine	0.99(d), 1.01(d,J=6.6 Hz), 1.04(d)
6	Ethanol	1.19(t), 3.65(q)
7	Fatty acid	1.25(s), 1.3(m)
8	Lactate	1.33(d), 4.11(q)
9	Alanine	1.48(d), 3.77(q)
10	Acetic acid	1.92(s)
11	N-Acetylaspartate	2.02(s)
12	Glutamate	2.06(m), 2.35(m), 3.76(m)
13	Methionine	2.13(s), 2.65(t)
14	O-Acetylcholine	2.14(s), 3.21(s),
15	Glutathione	2.16(m), 2.55(m)
16	Acetone	2.24(s)
17	GABA	2.31(t,J¼7.2 Hz), 3.01(t, J¼ 7.2 Hz)
18	Succinic acid	2.41(s)
19	α-Ketoglutarate	2.44(t), 3.01(t)
20	Citrate	2.67(d), 2.72(d,16.2 Hz), 2.55(d,16.2 Hz), 2.82(d)
21	Dimethylamine	2.73(s)
22	Asparagine	2.87(q), 2.95(dd), 4.01(q)
23	Creatine	3.03s, 3.93s
24	Creatinine	3.05(s)
25	Choline	3.22(s), 4.07(t)
26	Acetylcholine	3.23(s)
27	Taurine	3.26(t), 3.42(t)
28	Inositol	3.28(t,J=9.0 Hz), 3.54(dd,J=3.0,6.6 Hz), 3.63(t,J=9.6 Hz), 4.07(t,J=3.0 Hz)
29	Methanol	3.36(s)
30	Methyl phosphate	3.47(d,10.2)
31	Glycine	3.57(s), 3.68(s)
32	Glycerol	3.56(dd,J=9.6,3.6 Hz), 3.66(dd,J=11.4,4.2 Hz)
33	Phenylacetylglycine	3.68(s), 7.37(m), 7.43(m)
34	Glucose	3.90dd
35	Mannose	3.94d
36	Uridine	4.36(m), 5.90(d), 5.92(d), 7.88(d)
37	Guanosine monophosphate	5.94(d), 8.20(s)
38	Uridine diphosphate	5.97(s), 7.96(d)
39	Uridine monophosphate	6.00(m), 8.11(d)
40	Adenosine monophosphate	6.14(d), 8.14(d), 8.24(s), 8.27(s), 8.58(3), 8.61(s)
41	Fumaric acid	6.52(s)
42	Tyrosine	6.89(d), 7.19(d)
43	Tryptophan	7.33(d)
44	Hypoxanthine	8.20(s), 8.21(s)
45	Formic acid	8.44(s)
46	Formate	8.46(s)