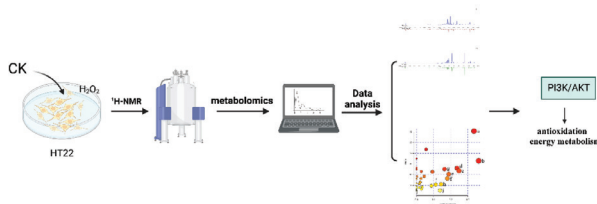


Protective effects of ginsenoside CK against oxidative stress-induced neuronal damage, assessed with ^1H -NMR-based metabolomics

Graphical abstract



Highlights

- A preliminary exploration of the endogenous metabolites involved in the effects of CK in damaged HT22 cells was conducted with ^1H -NMR.
- CK affects taurine, glycine, glutamate, and glutathione metabolism, according to metabolomic analysis.
- CK regulates ATP content in oxidatively damaged HT22 cells by upregulating the expression of components of the PI3K/AKT signaling pathway.

Authors

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In brief

CK affects energy-metabolism pathways and protects against neuronal oxidative stress through the PI3K/AKT signaling pathway.

Short Communication

Protective effects of ginsenoside CK against oxidative stress-induced neuronal damage, assessed with ¹H-NMR-based metabolomics

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ABSTRACT

Oxidative stress is an important pathogenic mechanism in degenerative diseases such as Alzheimer's disease. 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 oxidative stress damage induced by hydrogen peroxide in HT22 cells were investigated with ¹H nuclear magnetic resonance (¹H-NMR)-based metabolomics. The optimal CK concentration for decreasing oxidative stress damage in nerves was determined with MTT assays. CK (8 μM) significantly increased the HT22 cell survival rate after the model was established. Cell lysates were subjected to ¹H-NMR metabolomics, western blotting, and ATP assays for verification. Metabolic perturbation occurred in HT22 cells in the model group but not the control group. Twenty biomarkers were identified 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 the expression of components of the PI3K/AKT signaling pathway in HT22 cells. These findings demonstrated that CK prevents oxidative stress damage and protects nerves by regulating energy-metabolism pathways, such as those of taurine, glutamate, and other amino acids, thus providing a rationale for the use of CK in Alzheimer's disease treatment.

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 changes [1]. With the aging of human society, the number of patients with AD continues to increase. AD is caused by genetic, environmental, and lifestyle factors that affect neuronal cell degradation over time and severely affect quality of life in older people. Intensive research on the pathogenesis of AD is ongoing [2].

A major cause 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, thus 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, thus affecting the function and structure of biological molecules. ROS can also alter signal transduction and result 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 effects in traditional Chinese medicine such as increasing vitality, supporting the spleen and lungs, delaying aging, 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)-protopanaxadiol (CK)], one of the primary active metabolites of protopanaxadiol-type ginsenosides, is produced by the intestinal flora from ginseng diol saponins; moreover, CK has biological

properties, such as antiaging, neuroprotective, antioxidative, and anti-inflammatory effects [6]. In our previous study [7], we confirmed that CK has neuroprotective properties; however, no analyses of the antioxidative and neuroprotective effects of CK have used ^1H nuclear magnetic resonance ($^1\text{H-NMR}$)-based metabolomics.

Recently, metabolomic techniques, an important part of systems biology approaches, have been widely used to discover novel biomarkers [8, 9]. $^1\text{H-NMR}$ -based metabolomics has been applied to examine overall metabolic changes in organisms, thus enabling evaluation of the efficacy of traditional Chinese medicine formulations, including their pharmacological and toxicological mechanisms [10]. Cell metabolomics research has rapidly and intuitively described the metabolism of specific cell types with little interference, thus revealing the overall performance of physiological functions at the cellular level [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 and an important inducer of oxidative stress, which is used in the study of neurodegenerative diseases, such as AD, that are caused by oxidative stress [12]. We treated HT22 cells with H_2O_2 to establish an intracellular model of AD. In MTT assays, $8\ \mu\text{M}$ CK significantly increased the survival rate of HT22 cells (Figure 1). Thus, $8\ \mu\text{M}$ CK was used for metabolomics analysis.

We then investigated the potential protective effects of CK against H_2O_2 -induced changes in endogenous metabolites of HT22 cells with $^1\text{H-NMR}$ -based metabolomics. Differentially present metabolites in each treatment group were screened and identified, and the primary metabolic pathways were analyzed.

Analysis of the $^1\text{H-NMR}$ data combined with a comparison of previous studies enabled assignment of

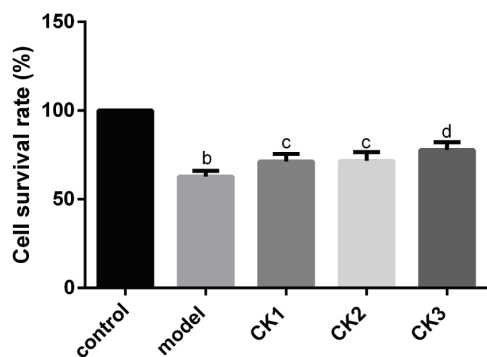


Figure 1 | Effects of CK treatment on cell growth.

Viability of cells treated with different concentrations of CK. Model: $400\ \mu\text{M}$ H_2O_2 , CK1: $4\ \mu\text{M}$ CK + $400\ \mu\text{M}$ H_2O_2 , CK2: $6\ \mu\text{M}$ CK + $400\ \mu\text{M}$ H_2O_2 , and CK3: $8\ \mu\text{M}$ CK + $400\ \mu\text{M}$ 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 \pm SD ($n = 8$).

signals of the main metabolite components in the three groups of cells (Figure 2, Table S1). The peak values of the $^1\text{H-NMR}$ spectra were normalized in MestReNova software, and the normalized peak data were imported into SIMCA-P software for partial least squares-discriminant analysis (PLS-DA) analyses.

The PLS-DA method removes interfering factors that are not associated with sample classification and maximizes the differences between groups. The results revealed differences in 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, thus indicating that the established PLS-DA model had good fit and predictability (Figure 3b). The CK group cluster was distinct from the model group cluster (Figure 3c), thereby indicating that the CK group differed from the model group.

The loading plot (Figure 3d) showed that the metabolites farther away from the origin could be considered candidate biomarkers. The metabolites with variable importance (VIP) values (VIP1 and VIP2) > 1 and $P < 0.05$ were highlighted as potential biomarkers. A total of 20 metabolites were considered as potential biomarkers that inhibit oxidative stress injury. Detailed information on 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 suggested that CK regulates taurine and hypotaurine metabolism, pyruvate metabolism, alanine aspartate and glutamate metabolism, and glycine serine and threonine metabolism (Figure 4a and b), thus decreasing 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 represents a key protein transformation node involved in amino acid and energy metabolism: it limits peroxidation, regulates glutamate metabolism, inhibits nerve cell apoptosis, and protects nerve cell function. Increasing evidence suggests that taurine-induced neuroprotection is mediated by the antagonism of glutamate-induced excitotoxicity [14]. Taurine content was much higher in the model groups than the control group, possibly as a compensatory response to antioxidant damage [15, 16]. Taurine content decreased after treatment with CK, thus indicating that CK decreased 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

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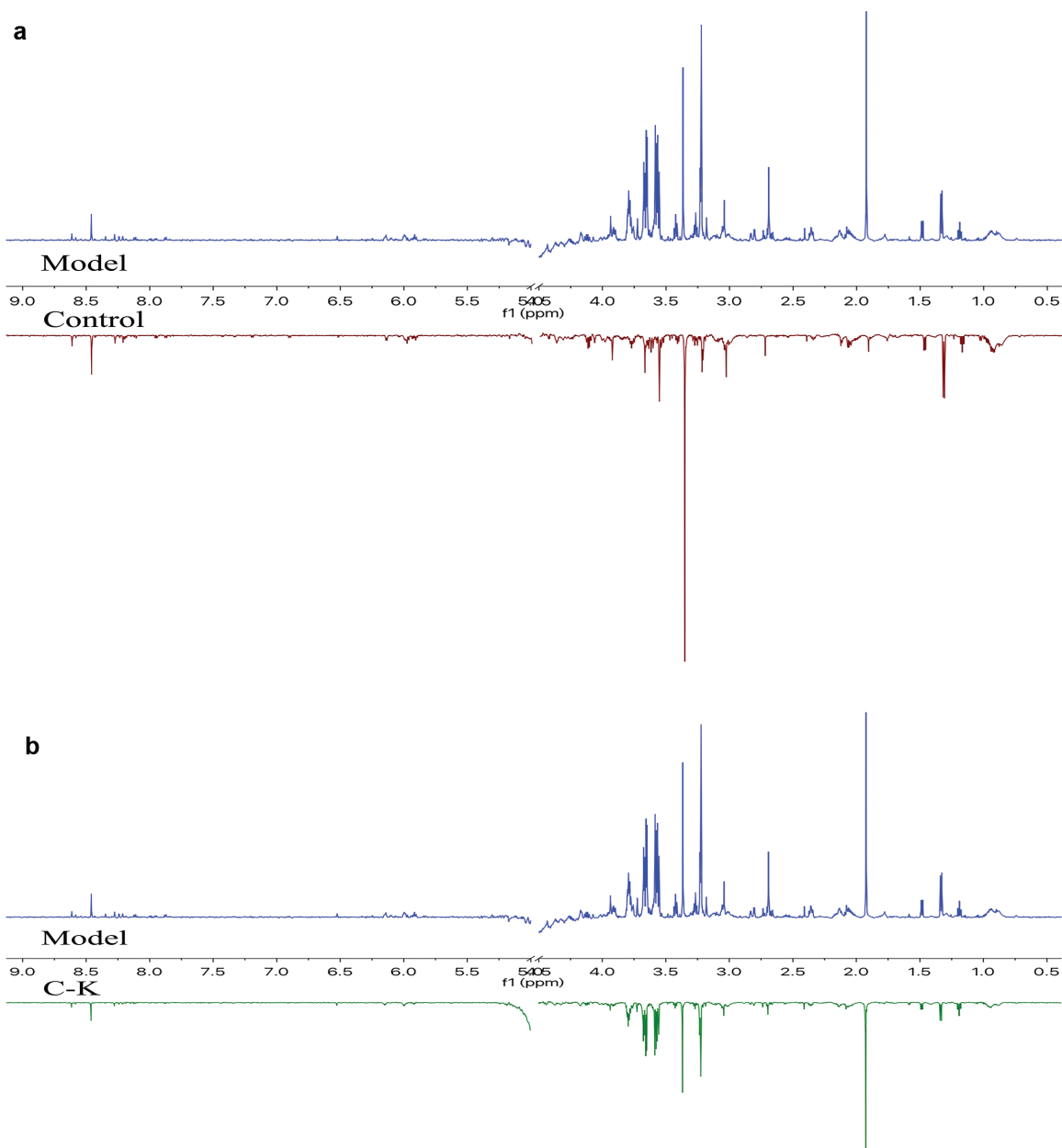


Figure 2 | Typical ¹H NMR spectra of HT22 cells.

(a) Control group and model group. (b) Model group and CK group.

brain and counteracts neurotoxicity due to peroxides such as H₂O₂ [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 that CK is involved in glycine

metabolism and increased glycine content, and thus plays a role in regulating glutathione metabolism and H₂O₂-induced oxidative decreasing damage in HT22 cells.

Glutamate, another essential precursor for glutathione synthesis, participates in glutathione

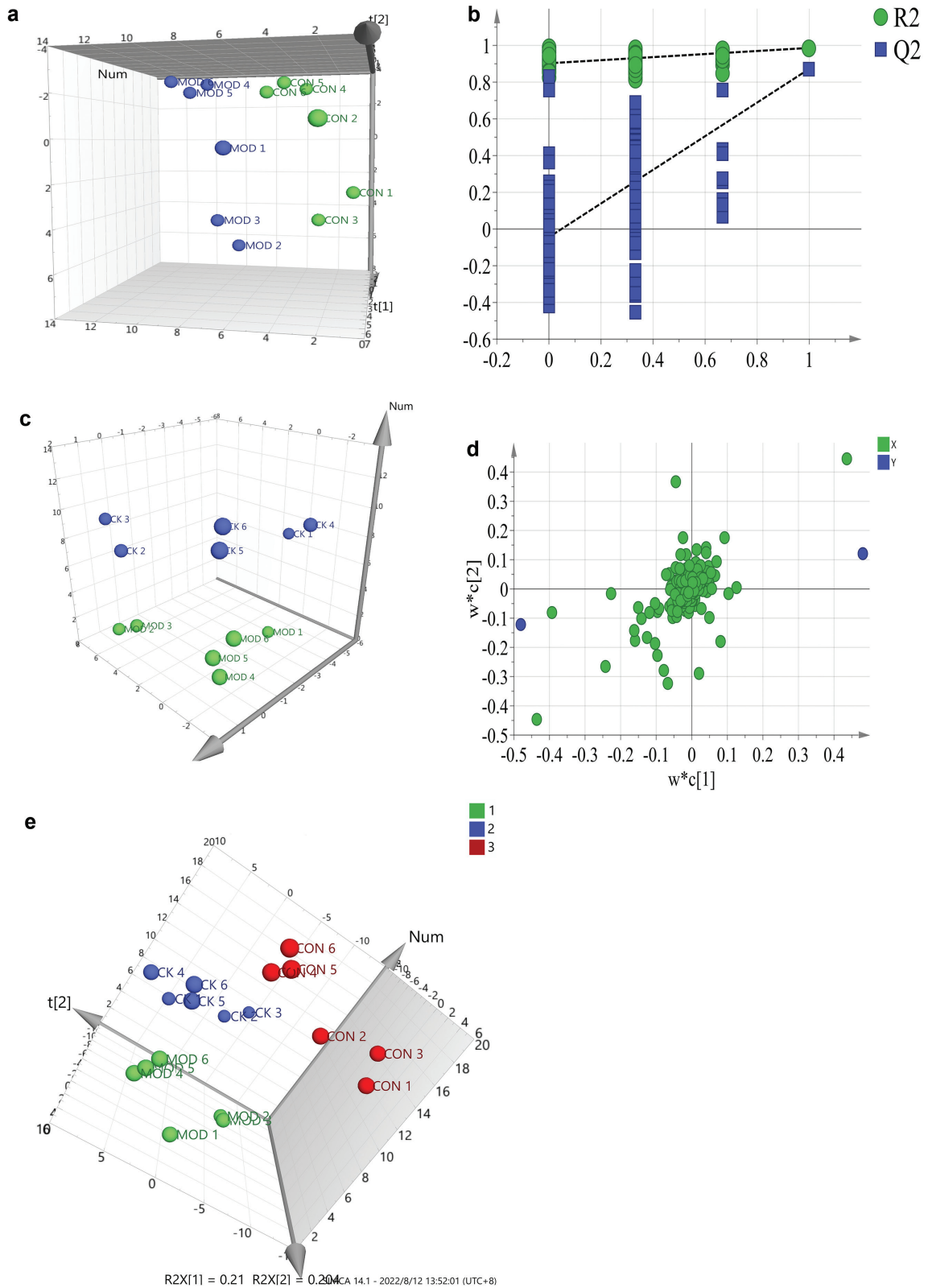


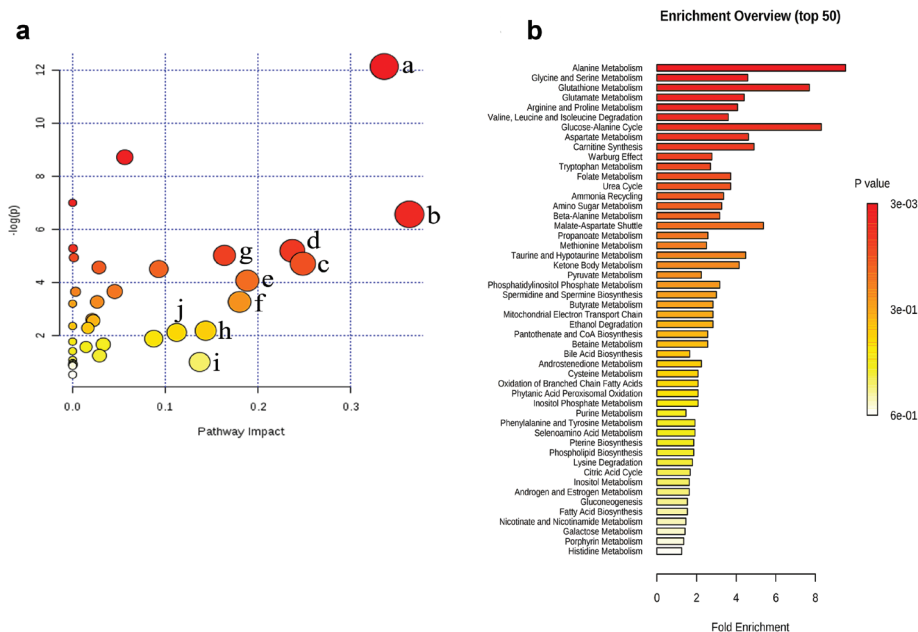
Figure 3 | (a) PLS-DA score plot of the control and model groups. (b) Permutation test of the control and model groups. (c) PLS-DA score plot of the CK and model groups. (d) Loading plot. (e) PLS-DA score plot of the control, model, and CK groups.

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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	Phenylacetylglucine	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.

**Figure 4** | Altered metabolic pathways in H₂O₂-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.

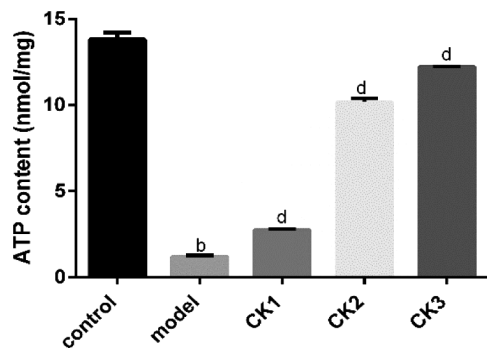


Figure 5 | Effects of CK on intracellular ATP levels of HT22 cells. Model: 400 μM H_2O_2 , CK1: 4 μM CK + 400 μM H_2O_2 , CK2: 6 μM CK + 400 μM H_2O_2 , and CK3: 8 μM CK + 400 μM H_2O_2 . ^b $P < 0.01$ vs. control group; ^d $P < 0.01$ vs. model group. Values represent mean \pm SD (n = 8).

metabolism [21] and is also an important neurotransmitter in the brain. 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 diminished glutamate levels in the brains of patients with AD [22], in agreement with the results of the present study suggesting that 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, we next

detected the expression levels of components of the PI3K/AKT signaling pathway through 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-3 β , increases GSK-3 β phosphorylation, thus inactivating GSK-3 β . The antagonistic effects of GSK-3 β activity influence central-nervous-system axon regeneration. Of note, inhibitors of GSK-3 β have been postulated to exert neuroprotective effects [25]. The present results indicated that CK increased the expression of components of the PI3K/AKT signaling pathway and improved regulation of the expression of proteins involved in energy-metabolism pathways (Figure 6).

We explored the effects of CK on endogenous metabolites in oxidatively damaged neurons *in vitro*. CK also has neuroprotective effects *in vivo* [26]. Whether CK can cross the blood-brain barrier is unknown. Karpagam et al. have shown that CK can cross the blood-brain barrier, according to ADMET assays [27], but further investigation is needed to determine whether this crossing also occurs *in vivo*.

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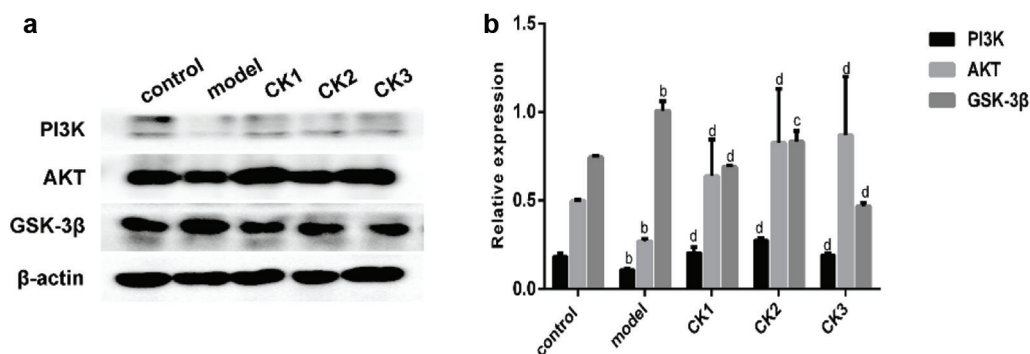


Figure 6 | Western blotting analysis of the expression of proteins associated with energy metabolism. Model: 400 μM H_2O_2 , CK1: 4 μM CK + 400 μM H_2O_2 , CK2: 6 μM CK + 400 μM H_2O_2 , and CK3: 8 μM CK + 400 μM H_2O_2 (a) and comparison of expression levels (b). ^b $P < 0.01$ vs. control group; ^c $P < 0.05$, ^d $P < 0.01$ vs. model group. Values represent mean \pm SD (n = 3).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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