Aspartic acid in the hippocampus: a biomarker for postoperative cognitive dysfunction

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Abstract

This study established an aged rat model of cognitive dysfunction using anesthesia with 2% isoflurane and 80% oxygen for 2 hours. Twenty-four hours later, Y-maze test results showed that isoflurane significantly impaired cognitive function in aged rats. Gas chromatography-mass spectrometry results showed that isoflurane also significantly increased the levels of N,N-diethylacetamide, n-ethylacetamide, aspartic acid, malic acid and arabinonic acid in the hippocampus of isoflurane-treated rats. Moreover, aspartic acid, N,N-diethylacetamide, n-ethylacetamide and malic acid concentration was positively correlated with the degree of cognitive dysfunction in the isoflurane-treated rats. It is evident that hippocampal metabolite changes are involved in the formation of cognitive dysfunction after isoflurane anesthesia. To further verify these results, this study cultured hippocampal neurons *in vitro*, which were then treated with aspartic acid (100 µmol/L). Results suggested that aspartic acid concentration in the hippocampus may be a biomarker for predicting the occurrence and disease progress of cognitive dysfunction.

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Introduction

Postoperative cognitive dysfunction is the deterioration of cognitive performance after anesthesia and surgery, and manifests as impairments in short-term memory, concentration, language comprehension, and social integration skills[1-3]. Thirty to forty-one percent of adult patients ranging from 18–60 years or older suffered from postoperative cognitive dysfunction at the time of discharge from hospital[4]. In patients older than 60 years, postoperative cognitive dysfunction was present in 25.8% of patients approximately a week after surgery and in 9.9% of patients 3 months after surgery[5]. Previous studies have shown that the occurrence of postoperative cognitive dysfunction is affected by many factors, including advanced age, low educational level, pre-existing cognitive impairment, alcohol abuse, and severity of coexisting illness[6-8]. However, the real cause for postoperative cognitive dysfunction is still unclear. Recently, we found that isoflurane (a general anesthetic agent) exposure impaired the cognitive function in aged rats[9]. Similar cognitive function impairment after general anesthesia was also reported in neonate, young, and adult rodents[9-13]. Further investigations showed differential modulation of isoflurane on neurogenesis in young and aged rats. Isoflurane increased early neuronal differentiation and progenitor proliferation of 60-day old rats[11]. However, it had no effects on neuro-

genesis in 16-month-old rats[14]. The pathophysiological mechanisms leading to the difference in the incidence rate and duration of postoperative cognitive dysfunction are still unclear. Additionally, the lack of biomarkers hinders the development of preventive and therapeutic strategies for postoperative cognitive dysfunction in clinical anesthesiology.

Metabonomics are defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"[15] and are of increasing interest in the determination of comprehensive metabolite profiles of biological body (biofluids, cells, tissues). Metabonomics incorporates whole systems biochemistry and thus provides crucial information on disease processes and gene function[16], and reflects the physiological or pathological status of an organism. Metabolite changes were reported in various neurodegenerative diseases, such as multiple sclerosis[17-19], Alzheimer's disease[20-23], Creutzfeldt-Jakob syndrome[24-25] and Huntington's disease[26-27]. Recently, Watanabe et al.[28] found that N-acetylasparate and myo-inositol concentrations in bilateral, especially left hippocampus changed in patients with amnestic mild cognitive impairment and Alzheimer's diseases. These findings suggested that metabolite changes played important roles in cognitive impairment. Gas chromatography-mass spectrometry has been proven to be a potentially useful

method for detection of small molecular metabolites based on its high sensitivity, peak resolution and reproducibility. Additionally, availability of the gas chromatography-mass spectrometry electron impact spectral library further facilitates the identification of diagnostic biomarkers and aids in the subsequent mechanistic elucidation of biological or pathological variations[29].

This study investigated the cognitive dysfunction of aged rats following isoflurane anesthesia, and detected the changes in hippocampal metabolites using gas chromatography-mass spectrometry. This study performed correlation analysis between changes in hippocampal metabolites and cognitive dysfunction in aged rats. These data will be useful in predicting the occurrence and development of postoperative cognitive dysfunction and will assist in elucidating the mechanisms underlying this phenomenon.

Results

Quantitative analysis of experimental animals
A total of 30 female Sprague-Dawley rats were randomly divided into two groups: isoflurane-treated group ($n = 20$) and control group ($n = 10$). Rats in the control group received 80% oxygen without administration of isoflurane for 2 hours. Rats in the isoflurane-treated group received 2 hours of 2% isoflurane and 80% oxygen. All rats underwent Y-maze testing 24 hours after anesthesia. After the last Y-maze test, rats were sacrificed for gas chromatography-mass spectrometry detection. All rats received similar care and were involved in the final analysis.

Physiological data during anesthesia
To observe changes in vital signs during anesthesia, heart rate, mean arterial blood pressure and saturation of oxygen in arterial blood (SaO_2) of rats in the isoflurane-treated group were monitored. The rats in the isoflurane-treated group showed stable mean arterial blood pressure, SaO_2 and heart rate during anesthesia compared with the rats in the control group. There were no signs of cyanosis, hypotension, respiratory depression or hypoxia (Table 1).

Isoflurane impaired cognitive functions of aged rats
In the Y-maze test, we agreed that an animal had mastered and remembered the maze when it entered the non-shock (illuminated) arm in nine out of 10 consecutive trials, thus an increase in the total number of trials required by the rats mastering the Y-maze indicated a decrease in learning and memory ability in the animals[5]. In the present study, the number of trials in the isoflurane-treated group was significantly higher than that in the control group (70.75 ± 15.30, *vs.* 45.4 ± 11.21; $P < 0.05$) at 24 hours after isoflurane anesthesia. This result suggested that isoflurane impaired cognitive functions of aged rats.

Isoflurane induced hippocampal metabolite changes of aged rats
Gas chromatography-mass spectrometry was validated prior to the analysis of the experimental samples, including stability and the reproducibility of sample preparation. A total of 47 interesting peaks determined by gas chromatography-mass spectrometry were qualitatively and quantitatively analyzed in the hippocampus of control and isoflurane-treated rats (Table 2). Compared with the control group, N,N-diethylacetamide levels (which were expressed as a ratio of the level of N,N-diethylacetamide to the level of internal control) in the isoflurane-treated group were 0.218 ± 0.042, which was 1.24 times that of the control group ($P = 0.017$). N-ethylacetamide was 0.419 ± 0.078, which was 1.24 times that of the control group ($P = 0.012$). Among the protein metabolites, in the isoflurane-treated group, aspartic acid was 0.344 ± 0.067, 1.32 times that of the control group ($P = 0.018$), and among the metabolites of carbohydrate, malic acid in the isoflurane-treated group was 0.096 ± 0.024, which was 1.26 times that of the control group ($P = 0.042$). Arabinonic acid was 0.032 ± 0.011, 1.93 times that of controls ($P = 0.029$). These results showed that isoflurane had significant effects on hippocampal metabolites in aged rats at 24 hours after anesthesia.

Hippocampal metabolites significantly correlated with cognitive function in isoflurane-treated aged rats
To identify a correlation between metabolites in the hippocampus and cognitive function in isoflurane-treated aged rats, correlation between trail number in the Y-maze tests and the relative concentration of five metabolites with significant changes was detected. Significant positive correlation was found between the Y-maze tests and aspartic acid ($r = 0.5$, $P = 0.005$, $r^2 = 0.25$), N,N-diethylacetamide ($r = 0.373$, $P = 0.042$, $r^2 = 0.139$), n-ethylacetamide ($r = 0.405$, $P = 0.026$, $r^2 = 0.164$), and malic acid ($r = 0.139$, $P < 0.001$, $r^2 = 0.019$). However, there was no significant positive correlation between trail number in the Y-maze test and arabinonic acid ($r = 0.065$, $P = 0.732$, $r^2 = 0.004$; Figure 1). These results showed that the hippocampal level of aspartic acid, N,N-diethylacetamid, n-ethylacetamide, and malic acid was correlated with cognitive function in isoflurane-treated aged rats.

Increased aspartic acid impaired cultured hippocampal neurons
To detect the toxic effect of increased aspartic acid on neurons, we first cultured hippocampal neurons for 7 days, which showed typical neuronal morphology and high level of microtubule-associated protein 2 expression (Figure 2A, C). Then we treated the cultured hippocampal neurons with 100 μmol/L aspartic acid. Compared with the control (Figure 2A, C), neurons treated with increased aspartic acid had fewer and shorter processes and smaller cell bodies (Figure 2B, D). Quantitative studies showed significant loss of neurons in the aspartic acid-treated group compared with the neurons in the control group (63.67% *vs.* 14.29%; $P < 0.05$).

Discussion
The present study evaluated the effects of isoflurane on metabolites in the hippocampus and its correlation with cognitive dysfunction. We demonstrated that isoflurane impaired the cognitive function of aged rats at 1 day after anesthesia

Table 1 Vital signs seen in rats during isoflurane anesthesia

Item	Isoflurane-treated group			Control group
	Before anesthesia	Induction of anesthesia	1 hour after anesthesia	
MAP (mmHg)	99.07±10.52	98.09±9.88	98.21±11.76	98.12±8.22
Heart rate (beats/min)	339.92±42.65	344.82±34.95	341.65±45.87	337.58±20.30
SaO$_2$ (%)	99.10±0.32	99.15±0.88	99.25±0.85	99.10±0.88
Appearance	Pink	Pink	Pink	Pink

Data are expressed as mean ± SD. There were 20 rats in the isoflurane-treated group and 10 rats in the control group. Paired *t*-tests showed that there was no significant difference within the isoflurane-treated group before induction, during induction, and 1 hour after anesthesia. Blood pressure, heart rate and SaO$_2$ changes between the control group and isoflurane-treated group were analyzed by independent sample *t*-tests. MAP: Mean arterial blood pressure; SaO$_2$: saturation of oxygen in arterial blood; 1 mmHg = 0.133 kPa.

with varying degrees. Meanwhile, isoflurane induced metabolites level shifts, especially N,N-diethylacetamide, n-ethylacetamide, malic acid and arabinonic acid, and aspartic acid. Moreover, the hippocampal levels of aspartic acid, N,N-diethylacetamide, n-ethylacetamide and malic acid positively correlated with cognitive function in isoflurane-treated aged rats. Increased level of aspartic acid could damage cultured hippocampal neurons.

Isoflurane is commonly used in clinical inhalation anesthetics and its effects on cognitive function have been well described[11-12, 14, 30-32]. For example, volatile anesthetics can impaire the cognitive function of rats[12, 14], but some also find volatile anesthetic neuroprotective effects and improvement of cognitive functions[14, 33-34]. Amyloid-beta (Aβ) production and/or accumulation are major pathological hallmarks of Alzheimer's disease[35]. Volatile anesthetics can also induce the Aβ oligomerization *in vitro*[36]. Isoflurane has been shown to induce caspase activation and apoptosis, interfere amyloid precursor protein processing, increase Aβ generation, aggregation and impair learning and memory[10, 37-46]. However, the mechanism of the Aβ oligomerization is unknown. Aspartic acid being isomerized aggregate in long-lived proteins of the aged people, especially in tooth, bone, cartilage, lens, and brain tissues[47], which is one of the most common post-translational modifications. The isomerization product is isoaspartic acid. Formation of isoaspartic acid is supposed to change protein structure and can change protein function and activity, or trigger aggregation[47-49]. Moreover, proteins containing isoaspartic acid may not fully degrade because that the isoaspartate residue hinders proteolytic degradation[50]. Isoaspartic acid is one of the direct mechanism related to the pathology of Alzheimer's disease. In our study, isoflurance induced the increased content of aspartic acid in the hippocampus and perhaps indirectly increased the isoaspartate by the isomerization, which finally led to the cognitive impairment of aged rats.

Although it has been known since the 1960s that aspartic acid has excitatory effects on neurons[51], it took almost 40 years for this amino acid to be considered as a classical neurotransmitter. It occurs in mammalian brain and has been implicated in the regulation of synaptic plasticity[52-53]. Aspartic acid is highly expressed in the whole brain during embryonic and perinatal periods, and strongly decreases during adulthood[54-56]. Nevertheless, in this study, aspartic acid in the hippocampus of the aged rats treated with isoflurane increased and was significantly correlated with cognitive impairment. Cui et al[57] also found that the aspartic acid and glutamine content in the rats' hippocampus increased with early spatial memory impairment, possibly by disrupting glutamate-N-methyl-D-aspartic acid receptor signaling and triggering aberrant tau hyperphosphorylation in the hippocampus.

To further testify our outcomes and observe the toxic effect of aspartic acid, this study cultured the hippocampal neuron *in vitro* and treated the hippocampal neuron with aspartic acid. A previous research showed that the concentration of d-Asp was 164 nmol/g in the cerebral hemisphere of the newborn rat[58]. Thus, our primary study first tested the dose-effects of aspartic acid (80, 100, and 150 μmol/L) on the cultured hippocampal neurons, and found that aspartic acid of 80 μmol/L was minimally excitotoxic, causing very little damage and death in these neurons. Moreover, aspartic acid of 150 μmol/L caused very serious loss of neurons. Therefore, our study detected the toxic effects of aspartic acid of 100 μmol/L, and demonstrated that the axonal and dendritic processes almost disappeared, even causing the loss of cell bodies in the group treated with aspartic acid. Increased aspartic acid directly impaired the neuron, which may be one of the underlying mechanisms of postoperative cognitive dysfunction induced by isoflurane.

Moreover, alterations in amino acid neurotransmitters in the brain are associated with cognitive impairments, degenerative diseases, and brain injury[59]. Aspartic acid concentrations in the patients with Alzheimer's disease have been found to alter, although these data are conflicting and further studies are warranted to corroborate these results. In 1990s, Fisher et al.[60] found aspartic acid concentration in the cerebrospinal fluid of patients with Alzheimer's disease was significantly higher than in normal cerebrospinal fluid. Integration of excitatory and inhibitory signals is a basic attribute of neuronal communication, and synaptic plasticity correlated with learning and memory requires the balance of excitatory and inhibitory neurotransmitters. This balance will be breakdown because of the increase in excitatory neurotransmitters and might have caused an imbalance between excitatory and inhibitory signals and decreased synaptic plasticity, resulting in cognitive impairment. Increased levels of endogenous aspartic acid induced an enhancement

Table 2 Changes in hippocampal metabolite induced by isoflurane in aged rats at 24 hours after anesthesia

Type of metabolites	Name of metabolites	Control group	Isoflurane-treated group	P value	Change (x-fold)
Metabolites of carbohydrate	Lactate	4.356±1.476	4.707±1.420	0.532	1.081
	Malic acid	0.076±0.024	0.096±0.024	0.042	1.263
	Isocitric acid	0.019±0.009	0.023±0.007	0.141	1.211
	Arabinonic acid	0.023±0.009	0.032±0.011	0.029	1.391
	Mannonic acid	0.117±0.054	0.132±0.037	0.381	1.128
Metabolites of protein	L-alanine	0.209±0.061	0.249±0.069	0.126	1.191
	Glycine	0.197±0.062	0.239±0.068	0.118	1.213
	Norvaline	0.034±0.008	0.040±0.011	0.128	1.176
	Leucine*	0.020±0.006	0.030±0.008	0.148	1.500
	L-isoleucine	0.027±0.007	0.030±0.008	0.378	1.111
	Proline	0.029±0.010	0.034±0.011	0.244	1.172
	4-Amino butyric acid	0.348±0.124	0.435±0.116	0.068	1.250
	Glyceric acid	0.008±0.004	0.008±0.007	0.895	1.000
	Serine	0.184±0.052	0.220±0.043	0.054	1.196
	Thereonine	0.106±0.026	0.119±0.029	0.247	1.123
	Aspartic acid	0.261±0.087	0.344±0.067	0.018	1.318
	4-Amino butyric acid	0.007±0.004	0.006±0.004	0.206	−1.143
Metabolites of lipids	Acetic acid	0.012±0.006	0.011±0.008	0.869	−1.083
	Bisoxyacetic acid	0.021±0.012	0.033±0.021	0.113	1.571
	Glycerol*	0.020±0.006	0.020±0.007	0.752	1.000
	Fumaric acid	0.013±0.004	0.017±0.005	0.077	1.308
	Creatinine enol	0.148±0.041	0.186±0.067	0.115	1.257
	n-Tetradecanoic acid	0.026±0.014	0.024±0.013	0.722	−1.077
	Pentadecanoic acid	0.016±0.008	0.013±0.008	0.275	−1.188
	Mannoonic acid	0.362±0.085	0.399±0.071	0.208	1.102
	Palmitic acid	0.750±0.132	0.786±0.133	0.487	1.048
	Linoleic acid	0.009±0.001	0.010±0.002	0.089	1.111
	Trans-9-octadecenoic acid	0.392±0.104	0.453±0.089	0.109	1.156
	11-Cis-octadecenoic acid	0.094±0.024	0.102±0.012	0.329	1.085
	Stearic acid	0.568±0.128	0.661±0.116	0.070	1.164
	Glycerol 1,2-dipalmitate	0.052±0.021	0.060±0.016	0.316	1.154
	Arachidonic acid	0.186±0.035	0.209±0.040	0.127	1.124
	11-Eicosenoic acid	0.021±0.008	0.024±0.005	0.225	1.143
	1-Monopalmitin	0.021±0.006	0.025±0.007	0.112	1.190
	Glyceryl 1,3-distearate	0.035±0.018	0.045±0.016	0.148	1.286
	Arachidonic acid	0.075±0.026	0.094±0.031	0.108	1.253
	1-Monopalmitin	0.107±0.014	0.109±0.025	0.841	1.019
	1-Monooleoylglycerol	0.034±0.007	0.038±0.009	0.171	1.118
	1-Monostearin	0.084±0.019	0.090±0.020	0.411	1.071
	5-Alpha-cholestane-3 beta	4.206±0.177	5.086±1.321	0.185	1.209
Others	N,N-diethylacetamide	0.176±0.041	0.218±0.042	0.015	1.239
	n-ethylacetamide	0.337±0.076	0.419±0.078	0.010	1.243
	Urea	0.506±0.168	0.604±0.160	0.130	1.194
	Phosphate	0.007±0.003	0.009±0.003	0.109	1.286
	Uracil	0.029±0.011	0.037±0.011	0.086	1.276
	Ascorbod acid	0.113±0.040	0.138±0.072	0.325	1.221
	Inositol	0.114±0.057	0.141±0.054	0.222	1.237

*: $\times 10^{-2}$. Data are expressed as mean ± SD, which were expressed as a ratio to the internal control. There were 20 rats in the isoflurane-treated group and 10 rats in the control group. Independent sample *t*-tests showed the levels of N,N-diethylacetamide, n-ethylacetamide, aspartic acid, malic acid and arabinonic acid in hippocampus of isoflurane-treated rats were significantly higher than those in the control rats ($P < 0.05$). Changes (x-fold): a negative number indicates that the comparison between the isoflurane-treated and control groups revealed a decreased multiple of the metabolite.

of the N-methyl-D-aspartate receptor-dependent long-term potentiation LTP and spatial memory abilities of mice. They accelerate the age-dependent decay of basal glutamatergic α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor transmission, synaptic plasticity and cognition in aged animals[61].
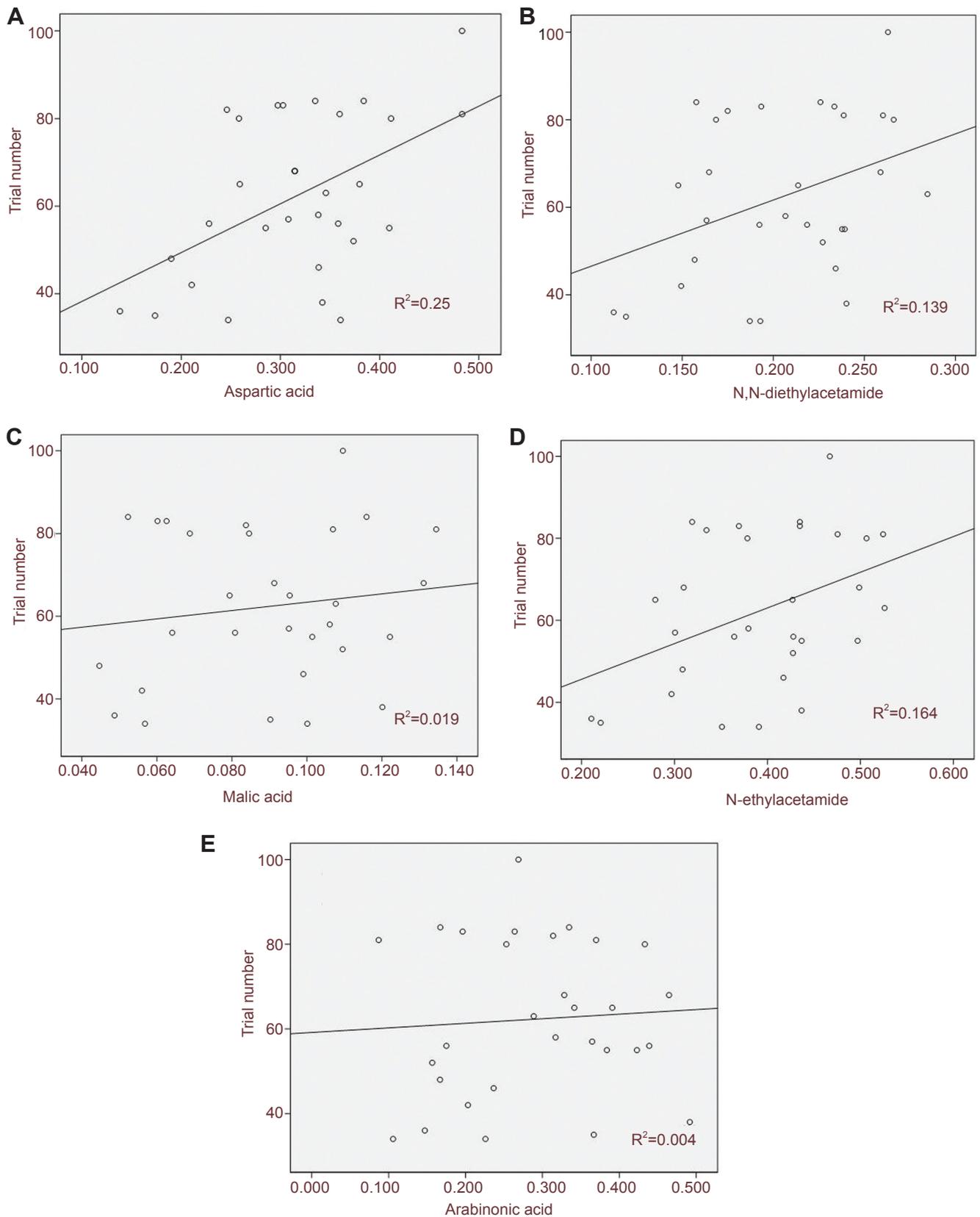
Figure 1   Correlation between trial number in the Y-maze and the relative concentration of hippocampal metabolites in isoflurane-treated aged rats.

The metabolites were expressed as a ratio to the internal control. Significant correlation was found between aspartic acid (A; $r = 0.5$, $P = 0.005$, $r^2 = 0.25$), N,N-diethylacetamid (B; $r = 0.373$, $P = 0.042$, $r^2 = 0.139$), malic acid (C; $r = 0.139$, $P < 0.001$, $r^2 = 0.019$), and n-ethylacetamide (D; $r = 0.405$, $P = 0.026$, $r^2 = 0.164$) and trial number in the Y-maze. No significant correlation was found between arabinonic acid (E; $r = 0.065$, $P = 0.732$, $r^2 = 0.004$) and trial number in the Y-maze using a linear regression model.
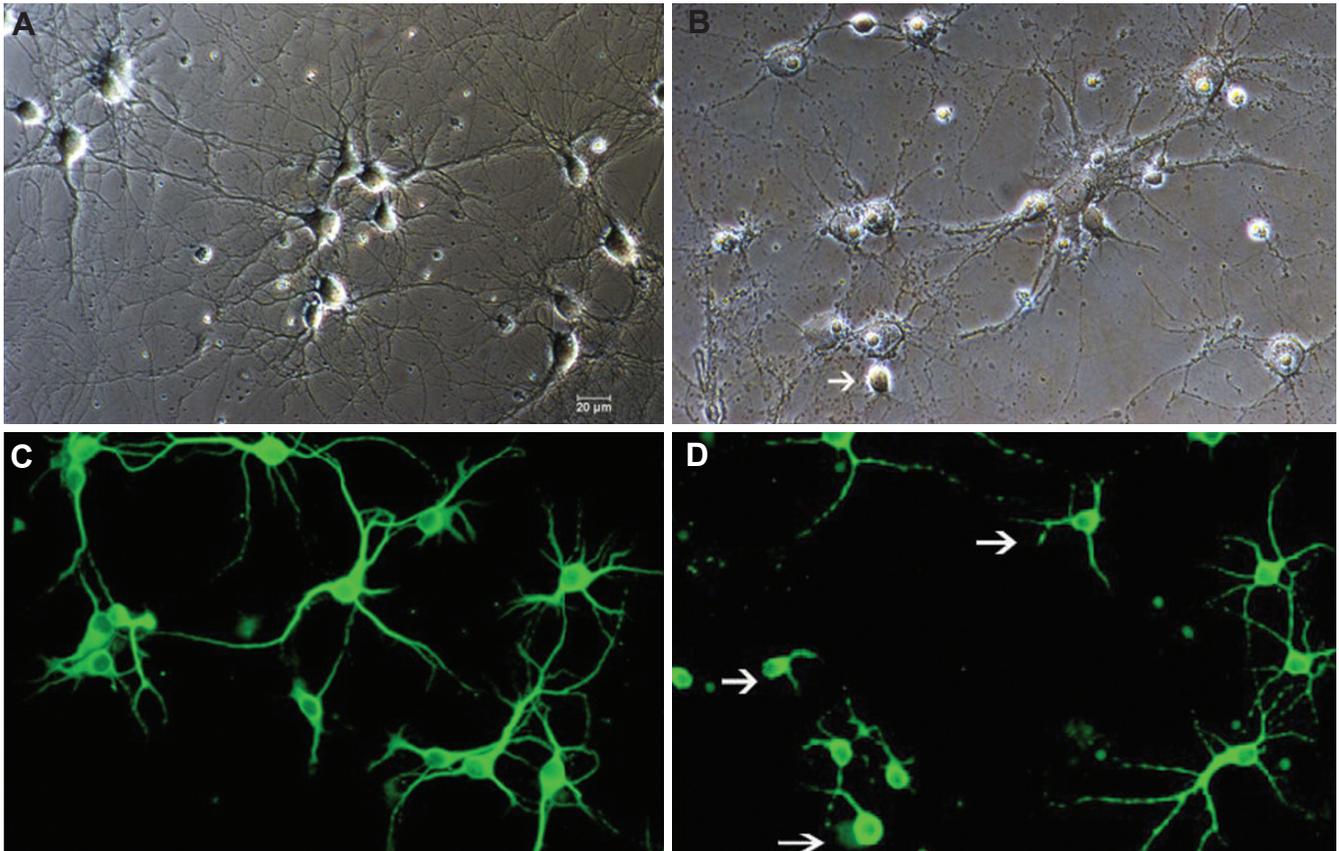
Figure 2 Effect of aspartic acid on cultured hippocampal neuron viability (× 200).
Representative pictures demonstrate the condition of cultured hippocampal neurons under phase-contrast microscope (A) and microtubule-associated protein 2 expression (C; immunofluorescence cytochemistry staining with FITC, epifluorescence microscope) in the control group. Neurons showed typical neuronal morphology. After treating with increased aspartic acid (100 μmol/L), parts of the neurons were lost and surviving neurons displayed shorter and fewer processes (arrows; B and D).

This study believed that hippocampal levels of aspartic acid could be used as biomarkers to predict the occurrence and progression of postoperative cognitive dysfunction in patients. However, the present study only used the female aged rat, and only detected the changes of cognitive function at the early stage after isoflurane. All the correlations are relatively weak. Thus further clinical study is still necessary for the actual effect size of aspartic acid as biomarkers in predicting the occurrence and progression of postoperative cognitive dysfunction in patients.

In addition, significant positive correlation was found between the Y-maze tests and N,N-diethylacetamide, n-ethylacetamide, and malic acid. Metabolic NADH shuttles promote the transfer of reducing cytosolic equivalents across the inner mitochondrial membrane into the mitochondria for oxidation to regulate the cellular redox and bioenergetic states. The α-glycerophosphate shuttle and malate-aspartate shuttle are the main parts of NADH shuttles. Here we found the changes of N,N-diethylacetamide, n-ethylacetamide and malic acid in aged rats with postoperative cognitive dysfunction. However, we still do not know the changed metabolites function in the occurrence and progression of postoperative cognitive dysfunction.

Materials and Methods

Time and setting
This experiment was conducted at the Experimental Center of the Third Xiangya Hospital of Central South University, China from January to September in 2010.

Materials
A total of 30 female Sprague-Dawley rats aged 22–24 months and weighing 300–500 g were used in this study for cognitive function evaluation. Five embryonic day 18 Sprague-Dawley rats were used to prepare hippocampal neuronal cultures. All animals were purchased from Hunan Agriculture College in China (license No. SCXK (Xiang) 2006-0001), and were housed under controlled environmental conditions (room temperature 22 ± 2°C, humidity 65 ± 1%) on a 12-hour light/dark cycle with free access to food and water. Experimental procedures were approved by the Animal Ethics Committee of the Central South University in China.

Methods
Establishment of the rat model of postoperative cognitive dysfunction after anesthesia
Anesthesia was given in a chamber flushed with 3% isoflurane

(Abbott, Chicago, IL, USA) and 80% oxygen. After the righting reflex was diminished, rats were then anesthetized through endotracheal intubation with 2% isoflurane and 80% oxygen with a respiratory rate of 39.19 ± 3.78 breaths/min. Pilot studies demonstrated that the $PaCO_2$ was 52.38 ± 7.74 mmHg at this time. The anesthesia was maintained at this concentration for 2 hours[12]. The concentration of isoflurane, oxygen, and carbon dioxide ($PaCO_2$) was monitored continuously by Datex equipment (Datex-Ohmeda, Madison, WI, USA). Mean arterial blood pressure was intermittently monitored with a rat tail blood pressure cuff. To objectively evaluate the effects of anesthesia, we measured $EctCO_2$, mean arterial blood pressure and heart rate, and respiratory rate in rats from the control and isoflurane-treated groups before, during and 1 hour after anesthesia induction. Rectal temperature was controlled at 37 ± 0.5°C. Rats in the control group were placed in a chamber flushed with 80% oxygen without isoflurane anesthesia.

Y-maze test

The Y-maze test is widely used to evaluate abilities of spatial learning and memory in rodents[4]. Cognitive functions were detected by an investigator who was blinded to intervention using the modified Y-maze apparatus 24 hours after delivery of isoflurane anesthesia or 80% oxygen. The behavioral tests were carried out in a separate room (temperature was approximately 22°C) and were conducted during the dark phase. The Y-maze was used to assess spatial memory. The apparatus consists of three opaque-black arms connected into a Y shape. The floor of the stem of the Y arm and one of the two branch arms contained electric wires through which an electric shock could be applied. The branch arm lacking wires remained illuminated throughout the test (because rats are typically neophobic, they prefer to avoid an illuminated area). During the test, rats were placed in the stem of the Y-maze. When animals received an electric shock, they entered into one of the two branch arms. Before the next trial, rats were manually returned to the stem of the Y-maze. The same procedure was repeated until the animal entered into the non-shocking arm in nine out of 10 consecutive trials. The total number of trials was counted, indicating cognitive function.

Sample pretreatment of hippocampal tissues

After the Y-maze test, animals were anaesthetized with 10% hydronium. Animals were exsanguinated by cardiac puncture within 15 minutes, and then the brain was removed from the skull, and sectioned midsagittally. Rat hippocampi were removed from the brain immediately and washed in ice-cold physical saline solution. The isolated hippocampi were stored at −80°C until use. Hippocampal tissues (30 mg) were weighed and homogenized with a T10 basic homogenizer (IKA, Staufen, Germany) for 30 seconds at 0°C. Chloroform (100 mL), methanol (200 mL), and water (100 mL) were added to extract metabolites from the hippocampus. Methanol (400 mL) was then added and the sample was vigorously vortexed for 30 seconds. The resulting fluid was transferred to a 2 mL GC sampling vial containing 30 mL internal stan-

dards (heptadecanoic acid-methanol 1 mg/mL) and vortexed again for 30 seconds. Methanol (500 mL) was subsequently added, homogenized and centrifuged at 16,000 r/min for 20 minutes at 4°C. The resulting supernatant was dried in a vacuum centrifuge concentrator before the derivatization.

Gas chromatography-mass spectrometry analysis

All gas chromatography-mass spectrometry analyses were double-blinded and performed on a Shimadzu GCMS-QP2010 gas chromatography quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Helium carrier gas was used at a constant flow rate of 1.0 mL/min. A sample of 1.0 μL was injected into a DB-5 ms capillary column (30 m × 0.25 mm inside diameter film thickness 0.25 μm) at a split ratio of 1:10. The column temperature was initially maintained at 70°C for 4 minutes, and then increased at a rate of 8°C/min from 70 to 300°C and held for 3 minutes. Mass conditions were as follows: ionization voltage, 70 eV; ionsource temperature, 200°C; interface temperature, 250°C; full scan mode in the 35–800 atomic mass unit mass ranges with 0.2 second scan velocity; detector voltage, 0.9 kV.

Hippocampal neuronal culture and aspartic acid intervention

Cell cultures were prepared from embryonic day 18 rats and cultured as described previously[62]. The pregnant dam was sacrificed using an approved method of euthanasia, and the uterus was dissected out and placed in a sterile Petri dish. Working in a laminar flow hood, the fetuses were removed from the uterus, and their brains were dissected out and placed in a dish containing Neurobasal Medium (Gibco, Carlsbad, CA, USA). The tissue needs to remain submerged at all times. Using a dissecting microscope (Olympus, Tokyo, Japan), the meninges was removed from the medial aspect of the cerebral hemispheres, and then the hippocampus was dissected out. The hippocampi were collected in a dish containing Neurobasal Medium. When all of the hippocampi were removed, they were placed in a 15-mL conical centrifuge tube, and a volume of 4.5 mL was made up with CMF-HBSS (Invitrogen, NewYork, CA, USA). Trypsin (0.5 mL of 2.5%) was added to this tube and incubated for 15 minutes in a water bath at 37°C. Trypsin solution was gently removed, with the hippocampi remaining at the bottom of the tube. CMF-HBSS (5 mL) was added to the hippocampi, and allowed to stand for 5 minutes at room temperature. This was repeated twice to allow residual trypsin to diffuse from the tissue. Final volume was made up to 2–3 mL with CMF-HBSS (Invitrogen). The hippocampi were then triturated five to 10 times through a regular pipette, and then five to 10 passes through a Pasteur pipette that had been flame-polished so that the tip diameter was narrowed by half. The suspension was forcefully expelled against the wall of the tube to minimize foaming. By the end of this process, no chunks of tissue were visible. The dissociated neurons were plated at a concentration of 150,000 cells per 60-mm dish in plating media [Neurobasal Medium (Gibco) + GlutaMAX-I supplement (Sigma, St. Louis, MO, USA) + B27 serum-free supplement (Gibco)]. Using a micropipette, add the desired number of cells to each of the dishes containing the polylysine-treated coverslips in

Neuronal Plating Medium. Approximately 1 week before the animal reaches the correct gestational day, start the glial feeder cultures by seeding about 1×10^5 cells per 60-mm dish. Replace the medium in 60-mm dishes with fresh Glial Medium every 3–4 days. Cultures are suitable for coculturing with neurons when they reach 40–70% confluence. After 3–4 hours, the dishes were examined to ensure that most of the cells have attached, then coverslips were transferred to dishes containing a glial feeder layer in N2 or Neurobasal/B27 medium. The coverslips were inverted so that the paraffin feet were resting on the bottom of the dish. At 3 days after plating, cytosine arabinoside (1-β-D-arabinofuranosyl-cytosine) was added to a final concentration of 5 μmol/L to curb glial proliferation. In this medium, glial cell growth at 5 days dropped to less than 1.0%, resulting in a nearly pure neuronal population. The medium was changed every 2 days and cells were maintained until day 7.

Aspartic acid (100 μmol/L, BBI, Brescia, Italy) was applied to hippocampal neurons cultured for 7 days. After incubating for 25 minutes, the medium was removed, and followed with three washes with Neurobasal Medium and further incubation for 24 hours.

Immunofluorescence cytochemistry and detection of neuronal viability

For immunostaining, cultured neurons were fixed at indicated times in PBS containing 4% paraformaldehyde/4% sucrose for 20 minutes at 37°C. The neurons were then permeabilized with 0.1% Triton X-100 (Sigma)/PBS for 5 minutes and blocked with 0.5% fish skin gelatin (Sigma)/PBS for 1 hour, both at 27°C. The cells were then stained for somatodendritic markers using polyclonal rabbit anti-microtubule-associated protein 2 antibody (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:200; Jackson Immuno Research Labs, Inc., West Grove, PA, USA) at 37°C for 2 hours. The samples were washed with PBS, dewaxed, and mounted on slide glasses. The stained samples were imaged under an epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 20 × objective lens. The images were obtained with Axiocam MRm CCD camera (Zeiss) and AxioVision software (Zeiss). The toxic effects of aspartic acid on the neurons were evaluated by Live/Dead Assay kit (L-3224; Molecular Probe, Eugene, OR, USA) and the percent of damaged neuron number/total neuron numbers was calculated[63]. Neuronal viability was evaluated and compared with cultured neurons not exposed to aspartic acid.

Statistical analysis

All analyses were performed using SPSS 17.0 software for Windows (SPSS, Chicago, IL, USA). Quantitative data were expressed as mean ± SD. Paired *t*-tests were used to examine the changes in blood pressure, heart rate and SaO_2 within the isoflurane-treated group before induction, during induction and 1 hour after anesthesia. Blood pressure, heart rate, SaO_2 and metabolite changes between the control group and isoflurane-treated group were analyzed by independent sample *t*-tests. Correlation studies between metabolites and cognitive impairment were analyzed through a linear regression model. A value of $P < 0.05$ was considered statistically significant.

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