Metabolite changes in the ipsilateral and contralateral cerebral hemispheres in rats with middle cerebral artery occlusion

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Graphical Abstract

Abstract

Cerebral ischemia not only causes pathological changes in the ischemic areas but also induces a series of secondary changes in more distal brain regions (such as the contralateral cerebral hemisphere). The impact of supratentorial lesions, which are the most common type of lesion, on the contralateral cerebellum has been studied in patients by positron emission tomography, single photon emission computed tomodraphy, magnetic resonance imaging and diffusion tensor imaging. In the present study, we investigated metabolite changes in the contralateral cerebral hemisphere after supratentorial unilateral ischemia using nuclear magnetic resonance spectroscopy-based metabonomics. The permanent middle cerebral artery occlusion model of ischemic stroke was established in rats. Rats were randomly divided into 1, 3-, 9- and 24-hour groups and the sham group.

Key Words: nerve regeneration; brain injury; cerebral ischemia; middle cerebral artery occlusion model; ischemic hemisphere; contralateral hemisphere; metabonomics; 1H nuclear magnetic resonance; lactate; choline; γ-aminobutyric acid; diaschisis; neural regeneration
Introduction
Cerebral ischemia is a common cerebrovascular disease with high rates of disability and mortality (Anuncibay-soto et al., 2016). Metabolic disturbance plays an important role in ischemic brain injury, and an understanding of the underlying mechanisms is essential for the development of effective treatments (Yang et al., 2012). Focal brain lesions can have major effects on distal brain regions. A network perspective suggests that physiological effects of brain injury are best assessed over entire networks rather than just locally at the site of structural damage (He et al., 2007; Honey and Sporns, 2008; Carter et al., 2010).

Following the seminal report on the phenomenon of diaschisis, defined as a functional inhibition of the brain distant from the original site of injury (Igarashi et al., 2001), researchers started to investigate changes in brain regions far from the ischemic cerebral, such as the cerebellum and thalamus. Diaschisis has been shown to involve perturbations in glucose and oxygen metabolism as well as decreases in cerebral blood flow in adjacent brain regions (Engler et al., 2004). Arango-Davila et al. (2016) evaluated transcallosal changes after local ischemic injury in rats using the neuronal nuclear marker NeuN to examine interhemispheric diaschisis. Magnetic resonance imaging demonstrated that remote changes post-stroke are readily measurable in patients (Yassi et al., 2015). Cerebellar diaschisis (diaschisis between the supratentorial lesion and the contralateral cerebellar hemisphere), the most common form of diaschisis in patients with infarction of the deep middle cerebral artery territory, has been previously investigated by various research groups (Nguyen and Botez, 1998; Liu et al., 2007a; Lin et al., 2009; Madai et al., 2011). Another type of diaschisis, interhemispheric diaschisis, has also been studied, but to a lesser extent. It remains unclear whether the metabolism of the contralateral hemisphere is affected by the ischemic side.

1H nuclear magnetic resonance (1H NMR) spectroscopy is able to detect molecules based on their chemical shift. Many metabolites can be detected in vitro by 1H NMR, and it has been used to study changes in brain biochemistry associated with ischemic neuropathologic processes (Graham et al., 1992). In the present study, using 1H NMR, we investigated whether metabolite changes in the ischemic hemisphere impact metabolite changes in the contralateral hemisphere in the rat middle cerebral artery occlusion (MCAO) model of brain ischemia. Metabolic analysis may be a valuable approach for understanding the biochemical mechanisms of stroke and the associated diaschisis.

Materials and Methods

Animals
Thirty-eight male Sprague-Dawley rats, weighing 250–320 g and 8–9 weeks of age, were purchased from the Shanghai Laboratory Animal Co., Ltd., Shanghai, China. The rats were regularly fed and allowed free access to water in a quiet room at 25–26°C and 70% humidity at the Experimental Animal Center of Wenzhou Medical University, China (license No. SYXX (Zhe) 2015-0009). This study was approved by the Ethics Committee of Wenzhou Medical University of China (wydw2015-0094).

Rats were randomly divided into the MCAO group (n = 28) and the sham group (n = 9). The MCAO group was subdivided into the 1-hour, 3-hour, 9-hour and 24-hour subgroups (n = 7 for each), according to the duration of cerebral ischemia.

Permanent MCAO surgery
The MCAO model was established as previously described with some minor modifications (Longa et al., 1989). Rats were anesthetized with chloral hydrate (0.3 mL/100 g) and then placed in the supine position. After disinfection of the skin, a midline incision of 3–4 cm was made along the neck. Then, the left common carotid artery, vagus nerve, external carotid artery and internal carotid artery were successively separated. The common carotid and external carotid arteries were ligated with a silk suture and then an aneurysm clip was placed across the internal carotid artery. A V-shaped cut was made on the common carotid artery with microscissors, and a tip-rounded 3.0 monofilament nylon suture (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was inserted into the stump of the external carotid artery. Mild resistance indicated that the filament was inserted 1.6–1.8 cm into the internal carotid artery and blood flow was blocked at the middle cerebral artery origin. The left common carotid artery was ligated at the proximal end to fix the nylon suture. Finally, the skin was sutured and each rat was transferred to a heating blanket to recover from anesthesia. Rats in the sham group were subjected to the same manipulation, but without insertion of the monofilament nylon suture.

Neurological score assessment
Neurological deficit was graded using Longa’s scoring system (Longa et al., 1989) blindly by one experimenter. The scoring scale was as follows: 0, no apparent neurological deficit; 1, contralateral forelimb flexion; 2, circling motion toward the paretic side when attempting to walk; 3, falling to the lateral side when pushed gently; 4, no spontaneous locomotion and depressed levels of consciousness. Rats with a neurological score of 0 were excluded from further experiments.

Preparation of samples, acquisition of 1H NMR spectra and data analysis
Seven rats from each group were decapitated at 1, 3, 9 and 24 hours after MCAO. Nine rats in the sham group were sacrificed as control. Both the left and right cerebral tissues were quickly removed, snap-frozen in liquid nitrogen and stored at ~80°C for further processing. The brain tissue was weighed and mixed with distilled water (0.85 mL/g), ice-cold methanol (4 mL/g) and chloroform (2 mL/g). The supernatant was extracted and lyophilized for approximately 24 hours. The metabolite mixture obtained was then weighed and dissolved in vitro for NMR spectroscopy.

All 1H NMR experiments were carried out on a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), with a spectral width of 12 kHz. The acquisition time was 2.66 seconds per scan, with an additional 8-second relaxation delay to ensure full relaxation.
The number of scans was 128. The spectra were zero-filled to 64 K, and an exponential line-broadening function of 0.3 Hz was applied to the free induction decay prior to Fourier transformation. The pre-processed NMR data were then imported into the SIMCA-P+ 12.0 software package (Umetrics, Umeå, Sweden) for analysis and visualization by multivariate statistical methods. Data were mean-centered, and quantitative values of metabolite relative concentrations were obtained. The data were Pareto-scaled prior to partial least squares-discriminant analysis (PLS-DA). Finally, scatter plots and loading plots were acquired. The metabolite concentrations were determined from the spectra and normalized to the weight of the freeze-dried metabolite mixture.

Statistical analysis
Spectra data were statistically analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean ± SD. Independent t-tests were used for comparisons between groups. A P-value of < 0.05 was considered statistically significant.

Results
Neurological assessment
Rats before MCAO surgery and those in the sham group had no neurological deficits, with a score of 0. The scores in the MCAO 1-, 3-, 9- and 24-hour groups ranged from 1 to 4.

1H NMR spectra
Representative 1H NMR spectra of cerebrum samples from rats in the sham and MCAO 1-, 3-, 9- and 24-hour groups are shown in Figure 1.

1H NMR spectra and PLS-DA in the ipsilateral (left) ischemic hemisphere
The first principal components in the ischemic cerebral hemisphere in the MCAO 1-, 3-, 9- and 24-hour groups could be clearly distinguished from the sham group. Differences in the first principal components were also obvious in the MCAO 1-, 3-, 9- and 24-hour groups. With lengthening of the ischemic period, the differences in the first principal components (i.e., between the 3, 9 and 24-hour groups and the 1-hour group) became increasingly significant, as shown in Figures 2 and 3.

Changes in concentrations of metabolites in the ipsilateral (ischemic, left) hemisphere
Metabolite concentrations in the left cerebral hemisphere at different ischemic time points (1, 3, 9 and 24 hours) were compared with the sham group. Concentrations of lactate, alanine, γ-aminobutyric acid (GABA) and glycine were all higher in the MCAO groups than in the sham group. Lac-

Table 1 Metabolite concentrations in the ipsilateral (left) hemisphere in each group

| Metabolites | Sham group | MCAO 1-hour group | MCAO 3-hour group | MCAO 9-hour group | MCAO 24-hour group |
|-------------|------------|-------------------|-------------------|-------------------|-------------------|
| Lactate     | 23.30±0.78 | 25.99±1.72*       | 29.55±4.45*       | 36.97±3.43*       | 39.82±0.82*       |
| Alanine     | 1.95±0.12  | 3.44±0.44*        | 4.01±0.22*        | 3.39±0.64*        | 5.15±0.80*        |
| NAA         | 1.49±0.20  | 14.93±0.38        | 14.16±0.44*       | 11.89±0.73*       | 8.20±1.23*        |
| GABA        | 6.40±0.30  | 7.58±1.09*        | 9.94±1.38*        | 7.14±1.25*        | 7.62±1.08*        |
| Glutamate   | 24.21±0.62 | 21.33±1.00*       | 21.12±1.37*       | 21.50±1.34*       | 19.31±0.97*       |
| Aspartate   | 3.00±0.14  | 3.02±0.32         | 2.45±0.26*        | 2.57±0.24*        | 2.43±0.20*        |
| Creatine    | 16.30±0.74 | 16.20±0.47        | 15.33±0.67*       | 14.69±0.77*       | 13.67±0.73*       |
| Choline     | 1.89±0.13  | 2.62±0.64*        | 2.60±0.38*        | 1.78±0.42*        | 2.50±0.33*        |
| Glycine     | 1.81±0.12  | 2.24±0.18*        | 2.74±0.26*        | 2.36±0.32*        | 2.82±0.21*        |

Values are expressed as the mean ± SD (n = 7 for each of the MCAO groups and n = 9 for the sham group; independent t-test). *P < 0.05, vs. sham group. MCAO: Middle cerebral artery occlusion; NAA: N-acetyl aspartate; GABA: γ-aminobutyric acid.

Table 2 Comparison of metabolite concentrations at various ischemic time points in the ipsilateral (left) hemisphere

| Metabolites | MCAO 1-hour group vs. sham group | MCAO 3-hour group vs. sham group | MCAO 9-hour group vs. sham group | MCAO 24-hour group vs. sham group |
|-------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Lactate     | 0.00 +11.57                      | 0.01 +26.83                      | 0.00 +58.67                      | 0.00 +70.90                     |
| Alanine     | 0.00 +76.83                      | 0.00 +105.90                     | 0.00 +74.21                      | 0.00 +164.81                    |
| NAA         | 0.71 –0.40                       | 0.00 –5.59                       | 0.00 –20.68                      | 0.00 –45.32                     |
| GABA        | 0.03 +18.37                      | 0.00 +55.26                      | 0.24 +11.63                      | 0.02 +19.00                     |
| Glutamate   | 0.00 –11.93                      | 0.00 –12.78                      | 0.00 –11.21                      | 0.00 –20.25                     |
| Aspartate   | 0.85 +6.69                       | 0.00 –18.20                      | 0.01 –14.28                      | 0.00 –18.98                     |
| Creatine    | 0.75 –0.62                       | 0.01 –5.94                       | 0.00 –9.87                       | 0.00 –16.12                     |
| Choline     | 0.02 +38.38                      | 0.00 +37.22                      | 0.35 –5.97                       | 0.00 +36.57                     |
| Glycine     | 0.00 +23.72                      | 0.00 +51.33                      | 0.01 +30.03                      | 0.00 +55.66                     |

Changes are shown as an elevation (+) or decline (–) in metabolite concentration in the MCAO groups compared with the sham group. 0.00 indicates P < 0.01. P < 0.05 is considered statistically significant. The significance of metabolite changes was determined by independent t-test (P < 0.05). MCAO: Middle cerebral artery occlusion; NAA: N-acetyl aspartate; GABA: γ-aminobutyric acid.
Table 3 Metabolite concentrations in the contralateral (right) hemisphere in each group

| Metabolites | Sham group | MCAO 1-hour group | MCAO 3-hour group | MCAO 9-hour group | MCAO 24-hour group |
|-------------|------------|--------------------|-------------------|-------------------|--------------------|
| Lactate     | 23.14±1.32 | 25.79±0.62         | 26.58±1.25        | 27.72±1.29        | 28.61±1.61         |
| Alanine     | 1.90±0.10  | 2.12±0.09          | 1.99±0.11         | 1.94±0.09         | 2.04±0.08          |
| NAA         | 14.92±0.61 | 15.09±0.47         | 15.29±0.47        | 14.77±0.34        | 14.41±0.49         |
| GABA        | 6.42±0.31  | 5.93±0.20          | 6.25±0.23         | 6.22±0.32         | 6.68±0.28          |
| Glutamate   | 24.21±0.92 | 22.51±0.85         | 23.75±0.86        | 24.86±0.55        | 23.63±0.73         |
| Aspartate   | 3.04±0.17  | 3.58±0.09          | 3.49±0.19         | 3.43±0.17         | 3.38±0.12          |
| Creatinine  | 16.42±0.58 | 16.19±0.50         | 15.95±0.58        | 15.84±0.70        | 15.82±0.32         |
| Choline     | 1.92±0.20  | 1.80±0.18          | 1.79±0.18         | 1.78±0.18         | 2.20±0.21          |
| Glycine     | 1.84±0.15  | 1.97±0.16          | 2.05±0.15         | 1.78±0.11         | 1.94±0.06          |

Values are expressed as the mean ± SD (n = 7 for each of the MCAO groups and n = 9 for the sham group; independent t-test). *P < 0.05, vs. sham group. MCAO: Middle cerebral artery occlusion; NAA: N-acetyl aspartate; GABA: γ-aminobutyric acid.

Table 4 Comparison of metabolite concentrations at the various ischemic time points in the contralateral (right) hemisphere

| Metabolites | MCAO 1-hour group vs. sham group | MCAO 3-hour group vs. sham group | MCAO 9-hour group vs. sham group | MCAO 24-hour group vs. sham group |
|-------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|             | P      | Variation | P      | Variation | P      | Variation | P      | Variation |
| Lactate     | 0.00   | +11.45    | 0.00   | +14.86    | 0.00   | +19.80    | 0.00   | +23.61    |
| Alanine     | 0.00   | +11.36    | 0.10   | +4.44     | 0.39   | +2.11     | 0.01   | +7.21     |
| NAA         | 0.54   | +1.11     | 0.18   | +2.48     | 0.55   | −1.02     | 0.07   | −3.44     |
| GABA        | 0.00   | −7.72     | 0.21   | −2.74     | 0.19   | −3.16     | 0.09   | +3.96     |
| Glutamate   | 0.00   | −7.04     | 0.29   | −1.92     | 0.12   | +2.65     | 0.18   | −2.39     |
| Aspartate   | 0.00   | +17.62    | 0.00   | +14.51    | 0.00   | +12.59    | 0.00   | +11.03    |
| Creatinine  | 0.39   | −1.41     | 0.11   | −2.82     | 0.07   | −3.50     | 0.01   | −3.66     |
| Choline     | 0.19   | −6.45     | 0.18   | −6.66     | 0.12   | −7.65     | 0.01   | −14.24    |
| Glycine     | 0.09   | +6.97     | 0.01   | +11.11    | 0.32   | −3.57     | 0.12   | +5.29     |

Changes are indicated as an elevation (+) or decline (−) in the concentration of the metabolite in the MCAO groups compared with the sham group. 0.00 indicates P > 0.01, P < 0.05 is considered statistically significant. The significance of metabolite changes was determined by independent t-test (P < 0.05). MCAO: Middle cerebral artery occlusion; NAA: N-acetyl aspartate; GABA: γ-aminobutyric acid.

## Changes in metabolite concentrations in the contralateral (right) hemisphere

Metabolite concentrations steadily increased along with increasing length of the ischemic period (from 25.99 ± 1.72 at 1 hour to 39.82 ± 0.82 at 24 hours). Alanine and glycine increased nonlinearly, and reached a peak (5.15 ± 0.80 and 2.82 ± 0.21) at 24 hours of cerebral ischemia. GABA concentrations reached a maximum (9.94 ± 1.38) at 3 hours after ischemia. Conversely, N-acetyl aspartate (NAA) and creatinine continually decreased. Glutamate and aspartate reached a minimum (19.31 ± 0.97 and 2.43 ± 0.20, respectively) at 24 hours of ischemia. Choline increased at 1 hour (2.62 ± 0.64), 3 hours (2.60 ± 0.38) and 24 hours (2.59 ± 0.33) of ischemia. These changes were all statistically significant (P < 0.05) (Tables 1 and 2).

**Discussion**

Damage induced by MCAO is not confined to the infarct; secondary injuries may spread to other areas with a normal blood supply, such as the cerebellum and contralateral cerebrum (Stenset et al., 2007). The term “transhemispheric diaschisis” was first introduced in 1987. Hoedt-Rasmussen et al. (1964) reported a bilateral reduction of hemispheric blood flow in patients with unilateral cerebral infarction. They found that hemispheric blood flow was reduced on the healthy side as well, and they hypothesized that unilateral infarction decreased metabolism in the contralateral hemisphere. Accordingly, we hypothesized that the infarct might trigger changes in metabolites in the hemisphere contralateral to the damage.

NMR-based metabonomics, combined with 1H NMR spectroscopy, is a novel approach for rapidly identifying changes in global metabolite profiles of biological samples and has been applied in disease studies, such as stroke and diabetes (Nicholson et al., 2002; Yang et al., 2012; Guan et al., 2013). Metabolites reflect the integrative information of cellular function, and understanding changes in neurochemical metabolites may help identify region-specific biomarkers and advance our understanding of the molecular pathogenesis of brain lesions (Shen et al., 2014). In the present study, we examined changes in metabolites in both cerebral hemispheres in rats with MCAO using a 1H NMR-based metabonomics approach.

In the present experiment, we found that the first princi-
The continuous increase in lactate may imply an activation of GABAergic receptors, possibly due to the increased production from glutamate by glutamate decarboxylase, which can proceed without functioning mitochondria, diminished choline likely reflects elevated membrane turnover, and it is a precursor of acetylcholine and phosphatidylcholine (Miller, 1991). There is evidence that increased choline can inhibit the release of glutamate. It is widely accepted that glutamate and glycine, combined with decreased glutamate and aspartate, are two major excitatory amino acids and they play an important role in the pathways leading to cell death. Inhibitory amino acids, such as GABA and glycine, can inhibit the release of glutamate. It is widely accepted that an imbalance between excitatory and inhibitory amino acids underlies cerebral ischemic damage (Kato and Kogure, 1999; Bogaert et al., 2000; Nishizawa, 2001; Wang et al., 2014).

GABA and glycine were also increased and remained elevated, compared with sham-operated rats, in the ipsilateral ischemic side. GABA levels probably increase due to a shift toward anaerobic glycolysis in viable cells that continue to metabolize glucose under locally hypoxic conditions (Graham et al., 1992).

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In the present study, we also observed increased GABA and glycine combined with decreased glutamate and aspartate in the ischemic hemisphere in the acute stage. Glutamate and aspartate are two major excitatory amino acids and they may play an important role in the pathways leading to cell death. Inhibitory amino acids, such as GABA and glycine, can inhibit the release of glutamate. It is widely accepted that an imbalance between excitatory and inhibitory amino acids underlies cerebral ischemic damage (Kato and Kogure, 1999; Bogaert et al., 2000; Nishizawa, 2001; Wang et al., 2014).

Choline is a constituent of the phospholipid membranes of cells and reflects membrane turnover, and it is a precursor of acetylcholine and phosphatidylcholine (Miller, 1991). Therefore, increased choline likely reflects elevated membrane synthesis and/or a higher number of cells (Castillo et al., 1996).

The concentration of metabolites in the contralateral cerebral tissues was also compared with the sham group in this study. The continuous increase in lactate may imply an increase in anaerobic glycolysis in the contralateral cerebral hemisphere. The increase in aspartate and glycine and the reduction in GABA and glutamate may indicate a change in the balance between excitation and inhibition in the contralateral hemisphere. It is likely that the excitability of the contralateral cerebral hemisphere is influenced by the ipsilateral ischemic hemisphere mainly via the corpus callosum (Imbroschi et al., 2015). Creatinine serves as a major energy source when ATP...
is lacking, helping to maintain energy supply in cells. The slight reduction in creatinine levels in the contralateral cerebrum may indicate a perturbation in energy metabolism caused by the ischemic injury. Because the two cerebral hemispheres are connected by the large mass of neural fibers forming the corpus callosum, this type of diaschisis is referred to as transcallosal diaschisis (Reggia, 2004). It is widely thought that the primary mechanism responsible for transcallosal diaschisis is a loss of excitatory inputs from the damaged cerebral hemisphere that are conveyed by the corpus callosum to the intact contralateral cerebral cortex (Berlucchia, 1983; Caselli, 1991; Fiorelli et al., 1991; Meyer et al., 1993). Animal models have also demonstrated that the contralateral effects of an acute hemispheric infarct are reduced or abolished by prior sectioning of the corpus callosum (Kempinsky, 1958; Meyer, 1982). Although changes in metabolite concentrations in the contralateral cerebral hemisphere were detected in the current study, the underlying cellular and molecular mechanisms remain unknown, and further studies are required.

In conclusion, ¹H NMR-based metabonomics is a powerful tool for analyzing metabolic changes in the ipsilateral and contralateral cerebral hemispheres in rats with ischemic injury. Our findings provide further support for transhemispheric diaschisis. However, further studies are needed to clarify the complex mechanisms underlying this phenomenon.

**Author contributions:** HCG, YJY and HZS contributed to experimental design. TZ, QH, ZLH, and JLL contributed to animal experiments. NZX, WJC, YZ and JLL contributed to sample collection and metabolomics data acquisition. LR, YW and SCC participated in data analysis, result interpretation and writing. All authors have read, revised and approved the final version of the paper.

**Conflicts of interest:** None declared.

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**Figure 2 PCA score plots derived from ¹H NMR spectra of extracts from the left (ipsilateral) cerebral tissue of rats in the MCAO (colorized) and sham (black) groups.**

**X-axis:** The first principal component; **Y-axis:** The second principal component. (A) PCA scores plot for the MCAO 1-h (red) and sham group; (B) PCA scores plot for the MCAO 3-h (green) and sham group; (C) PCA scores plot for the MCAO 9-h (orange) and sham group; (D) PCA scores plot for the MCAO 24-h (blue) and sham group. The 2 experimental groups in (A), (B), (C) and (D) can be clearly separated; each point represents a sample. MCAO: Middle cerebral artery occlusion; PCA: principal component analysis; h: hour(s).

**Research ethics:** The study protocol was approved by the Ethics committee of Wenzhou Medical University (wydw2015-0094). All efforts were made to minimize the number and suffering of the animals used in the experiments in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No. 85-23, revised 1986), and “Consensus Author Guidelines on Animal Ethics and Welfare” produced by the International Association for Veterinary Editors (IAVE). The article was prepared in accordance with the “Animal Research: Reporting of In Vivo Experiments Guidelines” (AR- RIVE Guidelines).

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