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The free fatty acid metabolome in cerebral ischemia following human mesenchymal stem cell transplantation in rats

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) have the potential to promote brain repair and improve recovery following stroke. We investigated changes in free fatty acids (FFAs) following intravenous human MSC (hMSC) transplantation into rats that had undergone transient middle cerebral artery occlusion (MCAo).
Methods: Rats were subjected to 2-hours MCAo, followed by intravenous transplantation of hMSC or phosphate-buffered saline (PBS) at one day after MCAo. All rats were sacrificed 5 days after MCAo. Metabolic profiling of free fatty acids (FFAs) level was assessed in plasma and brain from control rats (n=8), PBS-treated MCAo rats (n=6), and hMSC-treated MCAo rats (MCAo+hMSC, n=6).
Results: The levels of some FFAs in plasma and brain samples of the MCAo and MCAo+hMSC groups were significantly different from those of the control group. The percentage composition of myristic acid in plasma and those of myristic acid, linoleic acid, and eicosenoic acid in brain tissues of the MCAo+hMSC group were significantly reduced compared to those in the untransplanted MCAo group.
Conclusion: Our metabolic approach has provided insights into understanding the complexity of biochemical and physiological events that occur in ischemic brain injury and the transplantation effects of MSCs in stroke.

1. Introduction

Mesenchymal stem cells (MSCs) have recently been investigated as an attractive therapeutic tool for ischemic stroke and myocardial infarction [1–3] because of their plasticity and availability [4]. Following stroke in a rat model, MSCs have been found to ameliorate functional deficits by secreting neurotrophic factors [5,6], which reduce apoptosis and promote endogenous cellular proliferation [5,7]. In our previous clinical report, transplantation of autologous human mesenchymal stem cells (hMSC) in patients with ischemic stroke and multiple system atrophy showed the potential to aid functional recovery [2,8].

Identification of reproducible surrogate outcome measure of a transplantation approach would aid in monitoring therapeutic effects. Few studies have attempted to establish a system with which to monitor the therapeutic effects of stem cells in ischemic rats. For example, magnetic resonance imaging (MRI) has recently been used to monitor the therapeutic effects of stem cell transplantation in chronic ischemia in rats [9,10], and MRI provides high-resolution images, showing the fate and migration of transplanted stem cells. Transplantation of MSCs in stroke might elicit a multifaceted cascade of biochemical events both in brain and circulating plasma. Nevertheless, monitoring such biochemical and therapeutic effects by measuring changes in organic compounds has not yet been investigated using either clinical samples or in rat models of ischemia. The composition of free fatty acids (FFAs) is an important biochemical indicator of metabolic pathways in various pathological conditions [11–14]. In particular, changes in polyunsaturated fatty acid (PUFA) levels have been associated with the production of reactive oxygen species [15], destruction of the antioxidant system, and inflammation during ischemia [16–19].

The relative risk of FFA composition in serum samples has been studied in human cerebral ischemic events [17,20]. Iso et al. reported that ischemic stroke is associated with changes in FFA; a higher intake of fish and n−3 polyunsaturated fatty acids is associated with a

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reduced risk of ischemic stroke, suggesting that FFA may have a role in protecting against ischemic stroke *via* a specific pathway in lipid metabolism [21]. In addition, linoleic acid found in clinical blood samples may protect against ischemic stroke, possibly by decreasing blood pressure, reducing platelet aggregation, and enhancing the deformability of erythrocytes [17]. However, analyses of differences in FFA composition in serum and in brain [22], and monitoring of biochemical changes mediating the transplantation of hMSC have not yet been performed.

Accurate discrimination of normal and abnormal states by analysis of complex metabolic profiles often requires the use of computer-aided pattern recognition. We previously used a star symbol plotting as a method of visual pattern recognition and readily discriminated X-linked adrenoleukodystrophy patients from normal controls [23]. This allowed the comparative analysis of the very-long-chain fatty acid composition in plasma, and the same approach was also used in an FFA profiling analysis of plasma from virus-infected rats and control animals [24]. The present study aimed at establishing the pattern of metabolic alterations to enable biochemical monitoring of hMSC transplantation. We used previously described methods [25–27] to perform a metabolic analysis of the FFA composition of plasma and brain samples from rats that had been subjected to transient middle cerebral artery occlusion (MCAo), hMSC treated MCAo (MCAo+hMSCs) and sham rats (operated rats underwent all surgical procedures, except that cerebral ischemia was not produced).

2. Materials and methods

2.1. Transient MCAo animal model

The use of animals in this study was approved by the Animal Care and Use Committee of Ajou University, and all procedures were carried out in accordance with institutional guidelines. Sprague–Dawley male rats (250–300 g) were anesthetized with 4% isoflurane and maintained with 1.5% isoflurane in 70% N_2O and 30% O_2 using a face mask. Rectal temperature was maintained at 37.0–37.5 °C with heating pads. Transient MCAo was induced using a method of intraluminal vascular occlusion that has been modified in our laboratory [28]. A 4-0 surgical monofilament nylon suture with a rounded tip was moved from the left common carotid artery into the lumen of the internal carotid artery to block the origin of the MCAo. Two hours after MCAo, reperfusion was performed by the withdrawal of the suture to the tip of the common carotid artery.

2.2. Experimental groups

One day post MCAo or sham, animals were divided into 3 groups: sham operation+phosphate buffered saline (PBS) injection (n=8), MCAo+PBS injection (n=6), and MCAo+hMSC (n=6) injection. In the MCAo+hMSC group, 1×10^6 MSCs were injected intravenously 24 h after MCAo. Animals were not immunosuppressed after hMSCs transplantation. All animals were sacrificed 5 days after MCAo, blood was obtained by cardiac aspiration and plasma was isolated. Tissue extracts from the brain were prepared by homogenization in diethylpyrocarbonate (DEPC) water (Sigma). Fresh plasma and brain tissue samples were immediately frozen at −70 °C until analyzed. To exclude variations in diet or other environmental factors, all rats were fed the same diet and maintained in the same environment.

2.3. Human MSC culture

hMSCs were obtained from 20 ml aspirates from the *iliac crest* of normal human donors[2,7] as part of a protocol approved by the Scientific-Ethical Review Board of Ajou University Medical Center (AJIRB-CRO-05-126). Briefly, cells were incubated in 150 cm^2 rectangular canted neck cell culture flasks (Corning Inc. Life Sciences, Lowell MA) at 37 °C in 5% CO_2 for 1 day and non-adherent cells were removed by replacing the medium. The medium was then changed every 2 to 3 days until the adherent cells became 80% confluent. The cells were harvested with 0.05% trypsin and 0.53 mmol/l EDTA (Gibco, Invitrogen, Carlsbad, CA) for 5 min at 37 °C, replated in a flask, and cultured for an additional 3 to 5 days before they were harvested. Cells used in these experiments were harvested after six passages. The expression levels of MSC surface markers (CD105 and CD73) in hMSC were evaluated using flow cytometry (n=3; FACScan; Becton-Dickinson, Rurtherford, NJ). MSCs showed high expression levels of stem cell markers CD105 and CD73 (range, 95–99%; Supplementary Fig. 1).

2.4. 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining

Twenty-four hours after MCAo, rats were anesthetized with chloral hydrate, and brains were removed and immediately sectioned coronally into six slices with 2 mm thick in a rodent brain matrix (Harvard Instrument Inc., South Natick, MA). Brain slices were placed in 2% TTC (T-8887, Sigma, Germany) and incubated at 37 °C for 40 min in the dark. The brain slices were then stained with TTC, and replaced with 10% formalin. The stained sections were photographed and scanned using a flatbed color scanner [29].

2.5. Immunohistochemistry

Immunohistochemistry is the localization of antigens or proteins in cells of a tissue section exploiting the principle of antigen–antibody interactions that are visualized by a marker in biological tissues [30–32]. Animals were sacrificed 5 days post MCAo. Brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde solution. The brains were kept overnight in paraformaldehyde at 4 °C, and then embedded in 30% sucrose solution until they sank. For immunohistochemical staining, 30 μm thick coronal sections were cut using a cryostat (CM1800; Leica, Germany).

For immunohistochemistry, the sections were washed three times with PBS and nonspecific binding was blocked with 10% horse serum. Cells derived from hMSC were identified using morphological criteria and immunohistochemical staining with NuMA (human nuclei matrix antigen; Oncogen), which is present in donor cells, but not in host parenchymal cells. The total number of NuMA-positive cells in the forebrain (bregma −1 ~ 1 mm) was measured on ten sequential slides (150 μm interval) [28], and the number of NuMA-positive cells was calculated by summing those found on all ten slides.

2.6. Chemicals and reagents

The 24 fatty acid standards and triethylamine (TEA) were from Sigma (St. Louis, MO). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was from Pierce

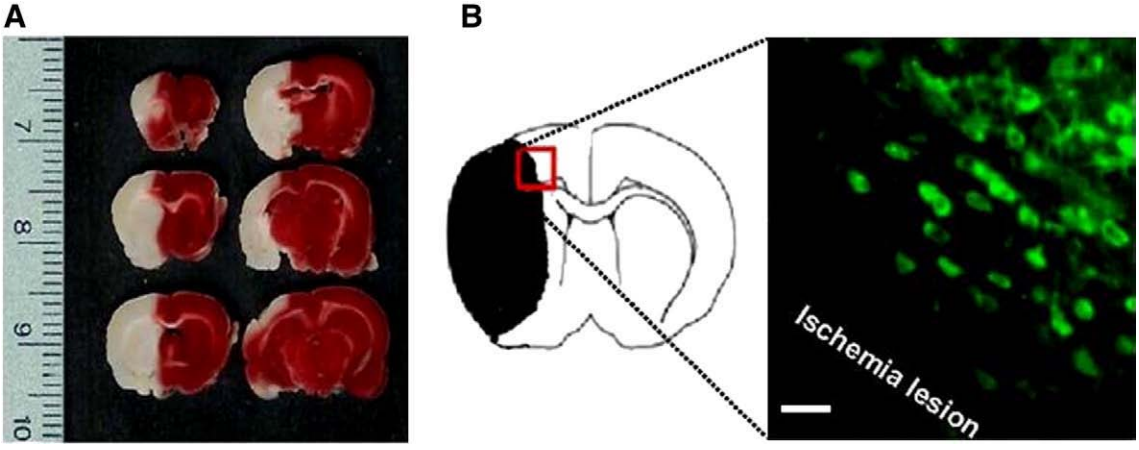

Fig. 1. TTC and hMSC staining. A. TTC-stained coronal brain sections. Brain sections were stained with TTC to visualize the ischemic lesions one day after transient middle cerebral artery occlusion (MCAo). The cerebral infarct is identified as white area, and normal brain is stained red. Lesion development was limited to the territory of the MCA. B. hMSC labeled 5 days post MCAo. A marked increase in NuMA-positive MSCs can be seen in the ischemic border zone (scale bar: 20 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Rockford, IL). Acetonitrile, toluene, diethyl ether, and dichloromethane of pesticide grade were from Kanto Chemical (Tokyo, Japan). To remove various interference including hydrocarbon, sodium chloride was from Junsei (Tokyo, Japan) and washed successively with methanol, acetone, dichloromethane, and diethyl ether, followed by drying under a vacuum (100 °C, 1 h). Sulfuric acid and sodium hydroxide were from Duksan (Seoul, South Korea).

2.7. Preparation of plasma samples

FFA in rat plasma samples were determined as their *tert*-butyldimethylsilyl (TBDMS) derivatives according to our previously described method [27,32]. Briefly, proteins were removed by adding 1 ml of acetonitrile to 0.1 ml of plasma containing 5.0 μg of pentadecanoic acid as an internal standard (IS). Following centrifugation, 1 ml of distilled water was added to the supernatant. Then, aliquot was adjusted to pH≥12 with 5.0 M sodium hydroxide and washed twice with 3 ml of diethyl ether. The aqueous phase was then acidified (pH≤2.0 with concentrated sulfuric acid) and saturated with sodium chloride, followed by extraction with diethyl ether (3 ml×2). Extracts were evaporated to dryness using a gentle stream of nitrogen. Dry residues containing FFA were reacted at 60 °C for 30 min with TEA (5 μl), toluene (20 μl), and MTBSTFA (20 μl) to form TBDMS derivatives. All samples were individually prepared in triplicate and directly examined by gas chromatography-mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode.

2.8. Preparation of brain samples

Rat brain samples were prepared for the FFA assay using our previously described method [27,32]. Total brain tissue placed in 5 ml of distilled water was homogenized at 30,000 rpm for 3 min in an ice-water bath using a rotor/stator-type tissue homogenizer (model Pro 200 Homogenizer, Oxford, UK). An aliquot of the homogenate equivalent to 20 mg of brain tissue, containing 5.0 μg of pentadecanoic acid as an IS was vortexed with 1 ml of acetonitrile for 3 min. The mixture was centrifuged at 15,000 rpm for 15 min to precipitate proteins. The supernatant layer was subjected to the aforementioned TBDMS reactions prior to GC-MS SIM analysis.

2.9. Gas chromatography-mass spectrometry

The GC-MS analysis in SIM mode was performed to quantitatively analyze FFA in rat plasma, using an Agilent 6890 gas chromatograph, interfaced to an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (SE-54 bonded phase; 25 m×0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA). Helium was used as the carrier gas at a constant flow rate of 0.5 ml/min. The injector, interface, and ion source were maintained at 260, 300, and 230 °C, respectively. Samples were introduced in the split-injection mode (10:1). The oven temperature was initially set at 100 °C for 2 min and programmed to rise at a rate of 3 °C/min to 260 °C, and finally at 20 °C/min to 300 °C (10 min). The mass range scanned was 50–750 u at a rate of 0.99 scans/s.

3. Result

3.1. Transplanted hMSCs were detected in the ischemic border zone

TTC staining result showed that infarcts were mostly located within the ipsilateral cortex and striatum (Fig. 1A). Cells derived from intravenously delivered hMSCs were identified in host rat brains using the human specific antibody NuMA (Fig. 1B). Transplanted hMSCs survived and were distributed throughout the ischemic damaged brain of recipient rats. Approximately 5% (45×10^3 to 50×10^3) of NuMA-positive MSCs had migrated into the ischemic tissue 4 days after the transplantation of hMSCs into the MCAo model. These results are consistent with a previous report in a similar model [28]. A few NuMA-reactive cells were also observed in several areas of the ipsilateral hemisphere including the cortex and striatum.

3.2. Plasma FFA composition in the rat model

The percentage composition of 24 FFA (μg/ml) was measured in plasmas obtained from 8 controls, 6 MCAo, and 6 MCAo+hMSC rats (Table 1). In the MCAo group compared to the control group, the levels of 15 FFA including caproic acid, were significantly increased (Student's *t*-test), whereas the levels of palmitic acid, linoleic acid, arachidonic acid, and eicosenoic acids were significantly reduced. In the MCAo+hMSC group compared to the control group, the levels of 15 FFA including caproic acid, were significantly increased, whereas the levels of palmitic acid, linoleic acid, and arachidonic acid were significantly reduced. In the MCAo+hMSC group compared to the MCAo group, the levels of 16 FFA were slightly decreased (Student's *t*-test), but the level of only myristic acid was significantly reduced to the level similar to controls. In contrast, the levels of oleic-, stearic-, arachidonic-, eicosenoic-, eicosanoic-, docosapentaenoic-, and behenic acid were slightly increased in the MCAo+hMSC group.

3.3. FFA composition in the brain of rat model

In the brain, the levels of 24 FFA (ng/mg) were measured (Table 2), however, the level of decenoic acid was lower than the quantification

Table 1
The percentage composition of 24 FFAs found in rat plasma samples from control rats, rats that had undergone transient middle cerebral artery occlusion (MCAo), and MCAo lesioned rats transplanted with human mesenchymal stem cells (MCAo+hMSC)

No.	Fatty acid	[a]Mean±standard deviation (*P* value[b])			
		Control (*n*=8)	MCAo (*n*=6)	MCAo+hMSC (*n*=6)	*P* value[c]
1	Caproic acid ($C_{6:0}$)	0.41±0.04	0.53±0.10 (0.005)	0.49±0.09 (0.02)	NS
2	Caprylic acid ($C_{8:0}$)	1.10±0.12	1.42±0.24 (0.003)	1.36±0.24 (0.01)	NS
3	Decenoic acid($C_{10:1}$)	0.52±0.06	0.68±0.11 (0.003)	0.65±0.12 (0.01)	NS
4	Capric acid ($C_{10:0}$)	1.10±0.13	1.41±0.23 (0.003)	1.35±0.23 (0.01)	NS
5	Lauric acid ($C_{12:0}$)	0.86±0.13	1.24±0.27 (0.003)	1.08±0.22 (0.02)	NS
6	Myristoleic acid ($C_{14:1}$)	0.42±0.05	0.55±0.09 (0.003)	0.53±0.10 (0.01)	NS
7	Myristic acid ($C_{14:0}$)	0.45±0.10	0.73±0.17 (0.001)	0.53±0.15 (0.1)	0.03
8	Palmitoleic acid ($C_{16:1}$)	5.02±1.23	6.12±1.88 (0.1)	6.06±2.15 (0.1)	NS
9	Palmitic acid ($C_{16:0}$)	20.44±0.81	19.65±0.30 (0.02)	19.50±1.17 (0.05)	NS
10	γ-Linolenic acid ($C_{18:3n6}$)	0.71±0.09	0.90±0.12 (0.003)	0.87±0.15 (0.01)	NS
11	Linoleic acid ($C_{18:2n6}$)	12.31±0.52	10.19±1.65 (0.002)	9.88±0.89 (0.00002)	NS
12	Oleic acid ($C_{18:1}$)	10.35±1.47	10.50±2.48 (0.4)	10.83±1.46 (0.3)	NS
13	Stearic acid ($C_{18:0}$)	24.17±1.83	24.59±3.29 (0.4)	24.98±1.87 (0.2)	NS
14	Arachidonic acid ($C_{20:4n6}$)	10.81±0.93	7.83±1.86 (0.001)	8.63±2.56 (0.02)	NS
15	Eicosapentaenoic acid ($C_{20:5n3}$)	1.90±0.22	2.24±0.36 (0.02)	2.21±0.28 (0.02)	NS
16	Eicosenoic acid ($C_{20:1}$)	0.82±0.06	0.74±0.08 (0.02)	0.76±0.09 (0.08)	NS
17	Eicosanoic acid ($C_{20:0}$)	0.24±0.02	0.26±0.03 (0.07)	0.27±0.05 (0.09)	NS
18	Docosahexaenoic acid ($C_{22:6n3}$)	2.66±0.30	3.22±0.37 (0.004)	2.98±0.16 (0.02)	NS
19	Docsapentaenoic acid ($C_{22:5n3}$)	1.59±0.15	1.93±0.25 (0.004)	1.95±0.32 (0.008)	NS
20	Erucic acid ($C_{22:1}$)	0.10±0.01	0.13±0.02 (0.004)	0.13±0.02 (0.02)	NS
21	Behenic acid ($C_{22:0}$)	0.09±0.01	0.09±0.02 (0.5)	0.10±0.02 (0.4)	NS
22	Nervonic acid ($C_{24:1}$)	1.29±0.15	1.67±0.27 (0.003)	1.61±0.31 (0.01)	NS
23	Lignoceric acid ($C_{24:0}$)	1.08±0.12	1.37±0.20 (0.003)	1.33±0.25 (0.02)	NS
24	Hexacosanoic acid ($C_{26:0}$)	1.55±0.19	2.02±0.33 (0.003)	1.94±0.38 (0.01)	NS

[a] Values shown are means±SD for the percentage composition by each FFA concentration (μg/ml).
[b] Student's *t*-test comparing the mean values of the MCAo group and the MCAo+hMSC group with those of the control.
[c] Student's *t*-test comparing the mean values of the MCAo and MCAo+hMSC groups.

Table 2
The percentage composition of 24 FFAs found in brain tissues from control rats, rats that had undergone transient middle cerebral artery occlusion (MCAo), and MCAo lesioned rats transplanted with human mesenchymal stem cells (MCAo+hMSC)

No.	Fatty acid	[a]Mean±standard deviation (*P* value[b])			
		Control (*n*=8)	MCAo (*n*=6)	MCAo+hMSC (*n*=6)	*P* value[c]
1	Caproic acid ($C_{6:0}$)	0.16±0.01	0.16±0.02 (0.4)	0.17±0.03 (0.1)	NS
2	Caprylic acid ($C_{8:0}$)	0.44±0.03	0.43±0.04 (0.3)	.47±0.07 (0.2)	NS
3	Decenoic acid($C_{10:1}$)	N.D.	N.D.	N.D.	
4	Capric acid ($C_{10:0}$)	0.43±0.03	0.42±0.04 (0.3)	0.45±0.07 (0.2)	NS
5	Lauric acid ($C_{12:0}$)	0.36±0.04	0.34±0.05 (0.2)	0.36±0.06 (0.4)	NS
6	Myristoleic acid ($C_{14:1}$)	0.17±0.01	0.17±0.02 (0.3)	0.18±0.03 (0.2)	0NS
7	Myristic acid ($C_{14:0}$)	0.15±0.01	0.23±0.05 (0.0003)	0.16±0.02 (0.2)	0.004
8	Palmitoleic acid ($C_{16:1}$)	2.52±0.11	2.43±0.10 (0.08)	2.49±0.05 (0.3)	NS
9	Palmitic acid ($C_{16:0}$)	18.49±0.38	17.32±0.54 (0.0002)	17.52±0.99 (0.01)	NS
10	γ-Linolenic acid ($C_{18:3n6}$)	0.27±0.02	0.26±0.03 (0.3)	0.29±0.04 (0.2)	NS
11	Linoleic acid ($C_{18:2n6}$)	0.53±0.05	0.73±0.13 (0.0007)	0.56±0.15 (0.3)	0.03
12	Oleic acid ($C_{18:1}$)	22.49±0.51	20.94±1.14 (0.002)	21.51±1.36 (0.04)	NS
13	Stearic acid ($C_{18:0}$)	24.31±0.79	26.21±1.56 (0.006)	25.41±1.17 (0.03)	NS
14	Arachidonic acid ($C_{20:4n6}$)	10.15±0.34	10.34±0.60 (0.2)	10.46±0.83 (0.2)	NS
15	Eicosapentaenoic acid ($C_{20:5n3}$)	0.55±0.06	0.55±0.05 (0.5)	0.62±0.15 (0.1)	NS
16	Eicosenoic acid ($C_{20:1}$)	2.56±0.20	2.89±0.07 (0.001)	2.69±0.23 (0.1)	0.03
17	Eicosanoic acid ($C_{20:0}$)	0.26±0.01	0.34±0.06 (0.002)	0.31±0.03 (0.003)	NS
18	Docosahexaenoic acid ($C_{22:6n3}$)	13.16±0.36	13.09±0.59 (0.4)	13.17±0.95 (0.5)	NS
19	Docsapentaenoic acid ($C_{22:5n3}$)	0.71±0.04	0.80±0.06 (0.002)	0.79±0.13 (0.05)	NS
20	Erucic acid ($C_{22:1}$)	0.17±0.01	0.20±0.01 (0.00009)	0.19±0.02 (0.03)	NS
21	Behenic acid ($C_{22:0}$)	0.16±0.01	0.18±0.03 (0.03)	0.18±0.03 (0.04)	NS
22	Nervonic acid ($C_{24:1}$)	0.69±0.04	0.72±0.04 (0.07)	0.72±0.10 (0.2)	NS
23	Lignoceric acid ($C_{24:0}$)	0.61±0.03	0.60±0.03 (0.3)	0.63±0.08 (0.3)	NS
24	Hexacosanoic acid ($C_{26:0}$)	0.66±0.04	0.64±0.06 (0.3)	0.70±0.11 (0.2)	NS

N.D.: not determined.
[a] Values shown are means±SD for the percentage composition by each FFA concentration (ng/mg).
[b] Student's *t*-test comparing the mean values of the MCAo group and MCAo+hMSC group with those of the control.
[c] Student's *t*-test comparing the mean values of the MCAo and MCAo+hMSC groups.

limit of the calibration curve and, therefore, the amount could not be determined. In the MCAo group compared to the control group, the levels of palmitic acid and oleic acid were significantly reduced, while the levels of myristic-, linoleic-, stearic-, eicosenoic-, eicosanoic-, docsapentaenoic-, erucic-, and behenic acid were significantly increased.

In the MCAo+hMSC group compared to the control group, the levels of 19 FFA were increased, and the increase of stearic-, eicosanoic-, docsapentaenoic-, erucic-, and behenic acid was statistically significant. In contrast, the levels of palmitoleic-, palmitic-, and oleic acids were slightly reduced. In the MCAo+hMSC group compared to the MCAo group, the levels of myristic-, 1inoleic-, and eicosenoic acid were significantly reduced.

3.4. Star pattern recognition analysis using plasma and brain samples

The levels of each of the 24 FFA in the MCAo and MCAo+hMSC groups were normalized to the corresponding mean values from the control group. These normalized values were used to create star graphs composed of 24 rays, so that the differences in the mean values among the control, MCAo, and MCAo+hMSC groups were presented

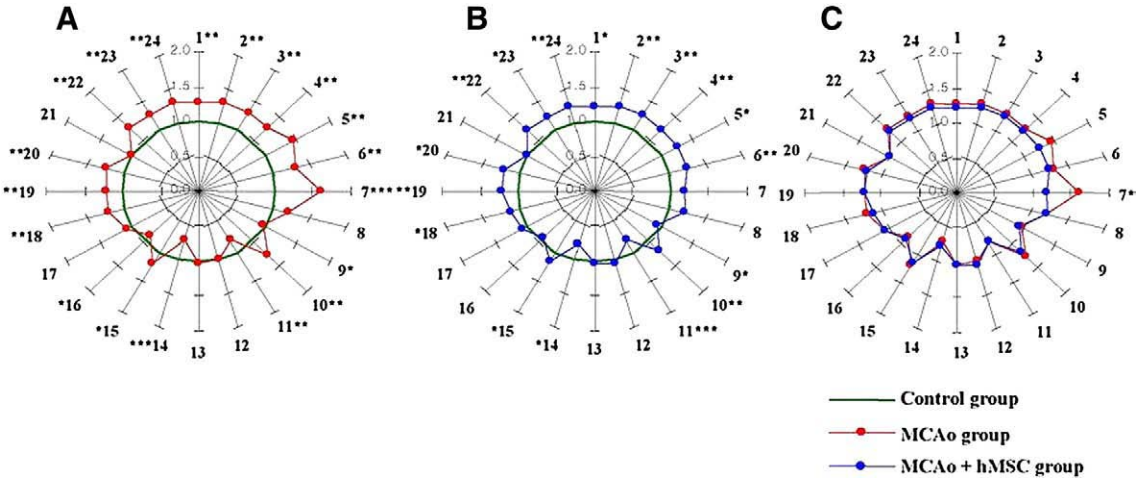

Fig. 2. Using star symbol plots overview of plasma metabolism changes. Star symbol plots of sham, tMCA+PBS, and tMCAo+hMSC rat based on the mean percentage composition of each of the 24 free fatty acids in plasma, normalized to the corresponding mean control values. (A) Control *vs.* MCAo, (B) control *vs.* MCAo+hMSC, and (C) MCAo *vs.* MCAo+hMSC. Ray numbers correspond to numbers inTable 1. *$P<0.05$, **$P<0.01$, ***$P<0.001$; Student's *t*-test at 95% confidence level. Rays: 1 = caproic acid ($C_{6:0}$), 2 = caprylic acid ($C_{8:0}$), 4 = capric acid ($C_{10:0}$), 5 = lauric acid ($C_{12:0}$), 6 = myristoleic acid ($C_{14:1}$), 7 = myrisic acid ($C_{14:0}$), 8 = palmitoleic acid ($C_{16:1}$), 9 = palmitic acid ($C_{16:0}$), 10 = γ-Linolenic acid ($C_{18:3n6}$), 11 = linoleic acid ($C_{18:2n6}$), 12 = oleic acid ($C_{18:1}$), 13 = stearic acid ($C_{18:0}$), 14 = arachidonic acid ($C_{20:4n6}$), 15 = eicosapentaenoic acid ($C_{20:5n3}$), 16 = eicosenoic acid ($C_{20:1}$), 17 = eicosanoic acid ($C_{20:0}$), 18 = docosahexaenoic acid ($C_{22:6n3}$), 19 = docsapentaenoic acid ($C_{22:5n3}$), 20 = erucic acid ($C_{22:1}$), 21 = behenic acid ($C_{22:0}$), 22 = nervonic acid ($C_{24:1}$), 23 = lignoceric acid ($C_{24:0}$), 24 = hexacosanoic acid ($C_{26:0}$).

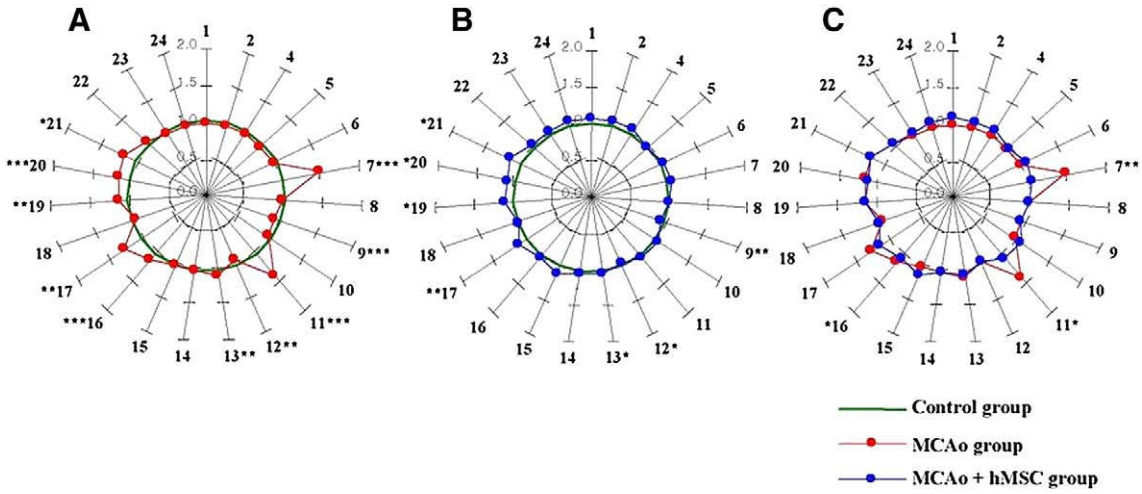

Fig. 3. Using star symbol plots overview of brain metabolism changes. Star symbol plots of sham control, tMCAo+PBS, and tMCAo+hMSC rat based on the mean percentage composition of 23 free fatty acids in the brain, normalized to the corresponding mean control values. (A) control *vs.* MCAo, (B) control *vs.* MCAo+hMSC, and (C) MCAo *vs.* MCAo+hMSC. Ray numbers correspond to numbers in Table 2. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; Student's *t*-test at 95% confidence level. The names of the 24 measured fatty acids are described in the legend of Fig. 2.

clearly. Visual star patterns were produced for plasma (Fig. 2) and brain samples (Fig. 3).

Plotting the normalized FFA values from plasma samples in the tetracosagonal shape was very informative. It clearly showed the elevation of FFA in multiples (ranging from 0.72 to 1.60) of the control mean values (Fig. 2). The levels of palmitic acid (No. 9), linoleic acid (No. 11), arachidonic acid (No. 14), and eicosenoic acid (No. 16) were lower in both the MCAo and MCAo+hMSC groups than in the control group (Fig. 2A, B). The amount of myristic acid (No. 7) in the plasma of the MCAo group was much greater than that of the MCAo+hMSC group (Fig. 2C).

FFA levels in brain samples from the MCAo and MCAo+hMSC groups were normalized to the corresponding mean control values, and they showed elevation of FFA in multiples (ranging from 0.93 to 1.51) of control values (Fig. 3). In the MCAo group, myristic acid was the most abundant, followed by linoleic acid (No. 11), whereas eicosanoic acid (No. 17) was the most abundant in the MCAo+hMSC group, followed by behenic acid (No. 21) compared to the control group (Fig. 3A, B). The levels of myristic acid (No. 7) and linoleic acid (No. 11) were much greater in the MCAo group than in the MCAo+hMSC group (Fig. 3C).

4. Discussion

To the best of our knowledge, this is the first demonstration of changes in the composition of FFAs in the plasma and brains of rats with cerebral ischemia following transplantation of hMSC. There is increasing evidence to indicate that hMSC promotes functional recovery in animal model of ischemic stroke. Several mechanisms have been suggested by which hMSC facilitates recovery from stroke; (a) replacement of infarcted tissue by true differentiation, spontaneous cell fusion, or both, and (b) up-regulation of endogenous recovery mechanism (neurogenesis or neuronal plasticity) related to the production of trophic factor released by the hMSC. Our present data suggest an alternate mechanism by which hMSC facilitates recovery from stroke, that is, elevated plasma or tissue levels of several FFA normalized after hMSC transplantation.

Generally, deficiencies in essential PUFA may be caused by an inflammatory reaction involving excessive oxidation, diminished antioxidant defenses, cellular damage, high rates of cell division, and cytokine-mediated reactions [22,33]. In particular, excessive amounts of oxygen radicals stimulate lipid peroxidation and depletion of essential PUFAs, thereby disturbing metabolic pathways during MCAo. Compared to the control group, the significant increase of endogenously synthesized saturated FFAs (except for palmitic acid) and monounsaturated FFAs in the plasma of the MCAo and the MCAo+hMSC groups may represent compensatory mechanism for the deficiency of available PUFAs (Table 1, Fig. 2). Moreover, the significant decrease in linoleic acid and arachidonic acid may explain the deficiency of PUFA. In the MCAo+hMSC group, saturated FFAs, which have cholesterol-increasing effects, and monounsaturated FFAs were slightly reduced compared to the MCAo group, with some exceptions (eicosenoic-, eicosanoic-, and behenic acid). Such reduction has been reported to lower cholesterol and has neuroprotective effects, because the antioxidant properties play a role in the defense mechanisms against oxidative stress [18]. However, there was no significant difference in the levels of PUFAs in the MCAo and MCAo+hMSC groups.

The *n*−6 and *n*−3 PUFAs present in the brain are not synthesized *de novo* in humans, and must either be obtained from diet or synthesized in the brain from linoleic acid (as the *n*−6 PUFA precursor) and α-linolenic acid (as the *n*−3 PUFA precursor) supplied in plasma [22]. In the MCAo group, the increased levels of linoleic acid and arachidonic acid may mediate and regulate inflammation, and this may explain the inflammatory reaction associated with MCAo. Moreover, these findings suggest that the brain obtained *n*−6 PUFAs, including linoleic acid, from plasma for a neuroprotective effect and to maintain brain function.

In the brain, the amount of *n*−6 PUFA linoleic acid was much higher in the MCAo group than in the control group, whereas the levels in the MCAo+hMSC group were not different from those in the control group (Table 2, Fig. 3). This suggests that the disturbance of PUFA metabolism by MCAo may be restored to a normal state after transplantation of hMSCs. Furthermore, this finding may explain the therapeutic amelioration of the MCAo lesion by hMSC transplantation. Therefore, the efficiency of stem cell therapy in preventing ischemic stroke may be enhanced by FFA intake.

The levels of myristic acid and eicosenoic acid in the brain of the MCAo group were significantly increased compared to those in the control and MCAo+hMSC groups. Most of the saturated and monounsaturated fatty acids that were increased in the MCAo group, compared with the control group, were reduced in the MCAo+hMSC group. Also, the saturated and monounsaturated fatty acids that were reduced in the MCAo group, compared to the control group, were increased in the MCAo+hMSC group. These findings suggest that changes in the levels of FFA may occur to compensate for the

deficiency of available PUFAs. The levels of FFA may recover to normal levels after hMSC transplantation into MCAo rats. Therefore, the efficiency of stem cell therapy seems to increase by using PUFAs for the prevention of ischemic stroke.

The normalized values of the 24 FFA in the MCAo and MCAo+hMSC groups were more informative than the raw values, because they express the elevation of FFA levels in multiples of the mean control values. The overall size of the MCAo group in the star symbol plots was larger than that of the MCAo+hMSC group; *i.e.*, the levels of FFA were generally greater in the MCAo group (Figs.2 and 3). The 2 star patterns were similar, except for the levels of lauric acid (No. 5) and myristic acid (No. 7) in plasma samples and of myristic acid (No. 7) and linoleic acid (No. 11) in brain samples. Thus, the 2 groups could be distinguished from each other. The control mean serves well as the control pattern for the MCAo and MCAo+hMSC groups and enables the visual comparison of the 2 groups with the control group. Star symbol plots drawn based on these 24 variables were very useful for visual pattern recognition of each group. The mean star plots representing the MCAo, MCAo+hMSC, and control groups were clearly distinguishable from one another. Therefore, it may be possible to use biochemical monitoring of FFAs to follow remitting and relapsing conditions, and to monitor therapeutic efficacy of hMSC transplantation following MCAo.

Several limitations deserve to be mentioned. First, the FFA levels were not measured serially in the present study. Serial assessment of FFA levels is needed to test the time-related impact of ischemia *per se* and hMSC transplantation. Second, our present study was focused on the changes in the FFA levels after stroke and hMSC transplantation, and the biochemical mechanisms by which hMSC controls FFA levels were not considered in the present study. Further studies on its link with stem cell biology are warranted.

In conclusion, our metabolic approach has provided insights into understanding the complexity of biochemical and physiological events that occur in ischemic brain injury and the transplantation of MSCs in stroke. A better understanding of FFA metabolism after MSC transplantation could lead to future development of effective strategies for stem cell therapy in cerebral ischemia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cca.2008.12.022.

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