Histochemistry of microinfarcts in the mouse brain after injection of fluorescent microspheres into the common carotid artery

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Abstract

The mouse model of multiple cerebral infarctions, established by injecting fluorescent microspheres into the common carotid artery, is a recent development in animal models of cerebral ischemia. To investigate its effectiveness, mouse models of cerebral infarction were created by injecting fluorescent microspheres, 45–53 µm in diameter, into the common carotid artery. Six hours after modeling, fluorescent microspheres were observed directly through a fluorescence stereomicroscope, both on the brain surface and in brain sections. Changes in blood vessels, neurons and glial cells associated with microinfarcts were examined using fluorescence histochemistry and immunohistochemistry. The microspheres were distributed mainly in the cerebral cortex, striatum and hippocampus ipsilateral to the side of injection. Microinfarcts were found in the brain regions where the fluorescent microspheres were present. Here the lodged microspheres induced vascular and neuronal injury and the activation of astroglia and microglia. These histopathological changes indicate that this animal model of multiple cerebral infarctions effectively simulates the changes of various cell types observed in multifocal microinfarcts. This model is an effective, additional tool to study the pathogenesis of ischemic stroke and could be used to evaluate therapeutic interventions. This study was approved by the Animal Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (approval No. D2021-03-16-1) on March 16, 2021.

Key Words: astrocytes; blood-brain barrier; common carotid artery; fluorescent microsphere; histochemistry; ischemia; microglia; microinfarcts; neuron; neurovascular unit; stroke

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Introduction

Ischemic stroke is caused by blockage of blood vessels in the brain leading to high levels of disability and mortality worldwide. Although our understanding of the structural characteristics of ischemic stroke has progressed, its pathological impact remains poorly understood (Moskowitz et al., 2010; Brundel et al., 2012; Smith et al., 2012; van Veluw et al., 2017; Shindo et al., 2020). Most of the research on the histopathological changes in ischemic stroke has been obtained from ischemic models. The ischemic model produced by intravascular injection of fluorescent microspheres into one side of common carotid artery (CCA) has recently been the preferred choice for the histochemical examination of the cellular changes in microinfarcts (Bere et al., 2014; Silasi et al., 2015; Tsukada et al., 2018; Balbi et al., 2019).

The breakdown of the blood-brain barrier (BBB) or the dysfunction of neurovascular unit (NVU) are considered the prime pathological characteristics of ischemic stroke (del...
Fluorescent microspheres

Fluorescent polyethylene microspheres made of polystyrene in a series of sizes were provided by Cospheric LLC (Santa Barbara, CA, USA). The diameter of the internal carotid artery is 121.29 ± 12.79 μm in mice (Qian et al., 2018) and we selected microspheres with 45–53 μm in diameter, into one side of the CCA. Fluorescent microspheres on the cerebral cortex were observed over the brain surface (Figure 1) using a fluorescence stereomicroscope (MVX10, Olympus, Tokyo, Japan). After examining the whole brain, each was cut into 80 μm-thick coronal sections with a freezing microtome (Thermo, Microm International GmbH, Walldorf, Germany) and the sections were collected in order in a six-hole Petri dish with 0.1 M PB (pH 7.4). The sites of lodged microspheres and microinfarcts in the brain were assessed according to a previous study (Paxinos and Franklin, 2001).

Materials and Methods

This study was approved by the Animal Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (approval No. D2021-03-16-1) on March 16 2021 and was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA).

Animals

Eight adult male C57BL/6 mice (6–8 weeks old, weighing 20–25 g) were provided by the Institute of Laboratory Animals, Chinese Academy of Medical Sciences, China (license No. SCXK (Jing) 2019-0010) for use in this study. All animals were housed in a 12-hour light/dark cycle with controlled temperature and humidity and allowed free access to food and water.

Fluorescent microspheres

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Fluorescent histochemical and immunohistochemical examinations

Fluorescent histochemical and immunohistochemical examinations were performed in four parts: (1) Nissl staining, (2) phalloidin + CD31, (3) Nissl staining + caspase 3, and (4) GFAP + Iba1 + 4′,6-diamidino-2-phenylindole (DAPI). Every sixth sections of the brain were used in each examination. For Nissl staining, the brain sections were directly stained by NeuroTrace™ 530/615 Red Fluorescent Nissl Stain (1:2000, Cat# N21482, Thermo Fisher, Waltham, MA, USA) in 0.1 M PB containing 1% Triton X-100 for 2 hours at room temperature. In phalloidin + CD31, Nissl staining + caspase 3, and GFAP + Iba1 + DAPI examinations, the sections were incubated in blocking solution containing 3% normal donkey serum (Cat# 017-000-121, Jackson ImmunoResearch, West Grove, PA, USA) and 1% Triton X-100 in 0.1 M PB (pH 7.4) for 0.5 hours at room temperature. The sections in these three parts were then incubated separately with primary antibodies: mouse anti-CD31 (1:1000, Cat# ab24590, Abcam, Cambridge, UK), rabbit anti-cleaved Caspase-3 (Asp175) (1:500, Cat# 9661, Cell Signaling, Danvers, MA, USA) and mouse anti-GFAP (1:1000, Cat# G3893, Sigma, St. Louis, MO, USA) and rabbit anti-Iba1 (1:1000, Abcam, Cat# ab178847) solution at 4°C overnight.

Surgical procedure for microsphere injection

In all experiments and surgical approaches, anesthesia was induced by intraperitoneal injection of pentobarbital sodium (50 mg/kg, Cat# 020402, Beijing Chemical Reagent Research Institute Co., Ltd., Beijing, China). Intravascular injection was performed as described previously (Bere et al., 2014; Silasi et al., 2015). In brief, a cervical incision was made to insert a polyethylene tube (PE-10 medical tube; Imamura Co. Ltd., Tokyo, Japan) into the right side of the CCA. A total of 100 μL of 1000 unit of microspheres (6–16 μg/mL) suspended in 5% Dextran T-40 (Cat# DB250, Solarbio Science & Technology Co., Ltd., Beijing, China) and then injected into each experimental animal through the polyethylene tube (n = 6). Vehicle controls received 100 μL 5% Dextran T-40 (n = 2). After injection, the polyethylene tube was withdrawn and the distal portion of the CCA was ligated simultaneously with surgical silk. The mice were kept warm with a hot blanket until they recovered from the anesthetic postoperatively.

Perfusions and sections

Six hours after the operation all mice were anesthetized again by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and transecrally perfused with saline followed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was dissected out and post-fixed in 4% paraformaldehyde in PB for 2 hours, then cryoprotected overnight in 25% sucrose. Fluorescent microspheres on the cerebral cortex were observed over the brain surface (Figure 1) using a fluorescence stereomicroscope (MVX10, Olympus, Tokyo, Japan). After examining the whole brain, each was cut into 80 μm-thick coronal sections with a freezing microtome (Thermo, Microm International GmbH, Walldorf, Germany) and the sections were collected in order in a six-hole Petri dish with 0.1 M PB (pH 7.4). The sites of lodged microspheres and microinfarcts in the brain were assessed according to a previous study (Paxinos and Franklin, 2001).
Statistical analysis

All brain sections of the six experimental mice were counted for microsphere distribution. Results are presented as the mean ± standard error of mean (SEM) with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The Kruskal-Wallis test was used to evaluate the distribution of fluorescent microspheres in different regions. The difference was considered statistically significant when \( P < 0.05 \).

Results

Distribution of fluorescent microspheres and microinfarcts

Six hours after intracarotid injection, the fluorescent microspheres were directly observed on the brain surface under the fluorescence stereomicroscope (Figure 1B). Additionally, the distribution of fluorescent microspheres was further mapped in the coronal sections of the brain (Figure 4). Microspheres were located in the arterioles, predominantly in the cortex but also in the striatum, thalamus, hippocampus, and other regions in the cerebral hemisphere ipsilateral to the side of injection (Figures 1–4). Microinfarcts were detected in the tissue near where the microspheres lodged in the occluded arterioles or their downstream branches (Figures 2–4). Corresponding to the locations of microspheres in the brain, the microinfarcts were scattered in the cortex, striatum, thalamus, hippocampus, and other regions. In the cortex, microinfarcts were found in typical wedge shape or column following the lodged microspheres in the penetrating arteriole at the pial surface (Figures 2–4). Microinfarcts in other cerebral regions varied in size and shape (Figures 2–4). A similar pattern of the distribution of fluorescent microspheres and microinfarcts was found in each microinfarct modelled mouse.

Changes in vascular integrity

The vascular integrity in the regions of microinfarcts was evaluated with phalloidin and CD31 (Figure 2). In control cases, phalloidin was expressed on the cerebral arterioles and their downstream branches, while CD31 was mainly present on the capillaries (Figure 2A and C). However, when the blood vessels were blocked with microspheres, expression of phalloidin followed the lodged microspheres and was found on the collapsed arterioles (Figure 2B and D). However, CD31 presented not only markedly on the capillaries but also at the location of the blocked blood vessels (Figure 2).

Neuronal degeneration and distribution

Neuronal alteration was examined using fluorescent Nissl and caspase 3 histological staining. Compared with the vehicle control, neuronal atrophy or cell loss was clearly demonstrated with Nissl labeling in the regions of microinfarcts (Figure 3). Cortical neurons in the layer 5 were typically pyramidal and distributed regularly in the vehicle controls (Figure 3), however, the cortical neurons in the region of microinfarcts were shrunken and their distribution was disorganized (Figure 3). Caspase 3 was expressed more strongly in the region of microinfarcts than that in the control case (Figure 3).

Astroglial and microglial activation

Astroglial and microglial activation was assessed with glial fibrillary acidic protein (GFAP) and Iba1 staining, respectively. In the vehicle controls, astrocytes and microglia presented with delicate cellular processes from their cell bodies and were observed throughout the brain tissue (Figure 4). When they were activated by the lodged microspheres, astrocytes gathered in the region of microinfarcts or along the wall of blocked vessels, presenting with thicker cellular processes (Figure 4). The microglia changed their form with enlarged cell bodies and short processes and, though scattered in the region of microinfarcts, accumulated particularly in peri-infarct regions (Figure 4).

Discussion

In this study, we described the pathological properties of multifocal microinfarcts in the mouse brain induced by the intracarotid injection of fluorescent microspheres. Taking advantage of this experimental model, our results provide histochemical views of the multicellular changes in the regions of cerebral microinfarcts at an early stage (Figure 5). This model of ischemic stroke may be beneficial in understanding the underlying mechanisms of multifocal microinfarcts and has the potential to assess novel therapeutic interventions of ischemic stroke.

Technological consideration

Various animal models have been used to investigate ischemic stroke, such as middle cerebral arterial occlusion and laser-induced occlusion of penetrating arterioles (Nishimura et al., 2010; Shih et al., 2013, 2015; Taylor and Sansing, 2013; Zhang et al., 2015; Shah et al., 2019). Although the middle cerebral artery occlusion model has been widely used to investigate ischemic stroke, it usually blocks the main trunk of the middle cerebral artery, leading to a large area of cerebral infarction (Shih et al., 2018; Shah et al., 2019). Laser treatment can induce microinfarcts at the brain surface through exquisitely controlling the location and timing of their onset, but it is limited to the surface of cerebral cortex (Shih et al., 2013; Taylor and Sansing, 2013; Zhang et al., 2015). Intracarotid injection of microspheres may complement the experimental models by mimicking the multifocal microinfarcts of ischemic stroke (Bere et al., 2014; Silasi et al., 2015; Tsukada et al., 2018; Balbi et al., 2019).

In the intracarotid injection of microspheres experimental model the microspheres tend to distribute in the hemisphere ipsilateral to the injection site, despite the blood supply of both cerebral hemispheres coming from the bilateral common carotid arteries via the circle of Willis. One possible explanation is that the injection and the ligation were carried out on the same side of CCA. Under these conditions, the blood supply from the contralateral CCA may dynamically prevent the entering of microspheres. This result is consistent with a previous study (Tsukada et al., 2018). In addition, the distribution of microspheres is determined by the diameter of microspheres themselves (Rapp et al., 2003, 2008; Reeson et al., 2018; Tsukada et al., 2018). This corresponds to the correlation between the number of microinfarcts and the diameter of the blood vessels to be blocked in the cerebral regions (Miyake et al., 1993; Bere et al., 2014; Silasi et al., 2015; Balbi et al., 2019). Since rather large-sized microspheres were used in the present study, they induced the blockage of larger diameter blood vessels, mostly branching from the middle cerebral artery, causing fewer infaracts but over a larger area. In comparison, infarcts induced by small-sized microspheres were limited to a smaller area but occurred in more numerous regions (Silasi et al., 2015). Although a similar model was successfully used in a previous study, only functionally multimodal imaging of the cerebral blood flow from the brain surface was examined (Bere et al., 2014). Importantly, our study additionally demonstrated histochemically the multicellular alterations after the multifocal microinfarcts.
Figure 1 | Injection and distribution of fluorescent microspheres.
(A) An illustration for the injection of fluorescent microspheres into the right side of the common carotid artery (CCA) of mice. (B) A representative photograph from the brain surface of mouse under the fluorescence stereomicroscope showing a typical distribution of fluorescent microspheres (green dots) lodged in blood vessels of the cerebral cortex. (C) A representative photograph from the coronal section of mouse brain with fluorescent Nissl staining showing the lodged microspheres (green dots) on the cortical surface and in the striatum. Scale bars: 2 mm in B, 1 mm in C. (D) The distributional percentage of microspheres in the different regions including the cerebral cortex (CC), striatum (Str), thalamus (Th), hippocampus (Hipp), and other regions. Data are expressed as mean ± SEM (n = 6) and were analyzed by Kruskal-Wallis test. *P < 0.05. ECA: External carotid artery; ICA: internal carotid artery; PPA: pterygopalatine artery.

Figure 2 | The vascular alteration induced by fluorescent microspheres.
(A–D) The representative photographs from the cerebral cortex (CC; A and B) and striatum (Str; C and D), showing the histological features of phalloidin (Pha, red, Alexa Fluor 568)- and CD31 (green, Alexa Fluor 488)-labeled blood vessels in the cases of control (A and C) and experimental model (B and D). (A1–D1) Magnified photographs from the box-indicated regions in panels A–D showing Pha- and CD31-labeled blood vessels in detail, respectively. CD31 labeling expressed more strongly in the regions of microinfarct than that of control. The vascular alteration in all model mice presented in a similar pattern (n = 6). Green dots in panels B/B1 (upper edge) and D/D1 (lower edge) are the lodged fluorescent microspheres. Scale bars: 200 μm in A–D, 100 μm in A1–D1.

Figure 3 | The neuronal degeneration in the region of microinfects.
(A, B) The representative photographs from the cerebral cortex showing the Nissl (green, NeuroTraceTM 500/525) and caspase 3 (red, Alexa Fluor 594)-labeling in the cases of control (A) and experimental model (B). (A1, B1) Magnified photographs from the box-indicated regions in panels A and B showing Nissl and caspase 3 labeling in detail, respectively. (A2, B2) Higher magnified photographs from panels A1 and B1. In contrast to the control, neurons are shrunken and caspase 3 labeling is expressed more strongly in the region of microinfarct. The neuronal degeneration in all model mice presented with a similar pattern (n = 6). The green dot in panel B is a lodged fluorescent microsphere near the hippocampus. Scale bars: 200 μm in A–H, 50 μm in A1–H1. DAPI: 4′,6-Diamidino-2-phenylindole.

Figure 4 | The astroglial and microglial activation in different regions of microinfarcts.
(A–H) The representative photographs from the cerebral cortex (CC, A and B), striatum (Str, C and D), hippocampus (Hipp, E and F), and internal capsule (IC, G and H) showing the glial fibrillary acidic protein (GFAP, red, Alexa Fluor 594)-labeled astrocytes and ionized calcium binding adapter molecule 1 (Iba1, green, Alexa Fluor 488)-labeled microglia in the cases of control (A, C, E, G) and experimental model (B, D, F, H). (A1–H1) Magnified photographs from the box-indicated regions in panels A–H showing the GFAP- and Iba1-labeling in detail. In the controls, the resting astrocytes and microglia were delicate in form and were distributed evenly in the different regions. In contrast, active astrocytes and microglia in the region of microinfarcts presented with enlarged cell bodies and the thicker cellular processes gathering around the wall of blocked vessels or scattered within the region of the microinfarcts. The astroglial and microglial activation in all model mice presented with the similar pattern (n = 6). DAPI: 4′,6-Diamidino-2-phenylindole.

Figure 5 | A simplified illustration of vascular and cellular alteration of microinfarct induced by a lodged fluorescent microsphere in the cerebral blood vessel.
In contrast to the normal area, the pathological alteration in the ischemic area includes the vascular blocking, neuronal degeneration, and astrogial/microglial activation.
Technological and design limitations

Despite the technological advantage that fluorescent microspheres are more easily identified in the blocked vessels in the brain, it should be noted here that these microspheres are made from polystyrene that can be dissolved by xylene. Therefore, conventional staining methods involving treatment with xylene cannot be used on brain sections with lodged microspheres. Instead, fluorescent histochemical or fluorescent immunohistochemical staining is recommended. In addition, it should be emphasized that the pathological changes that we observed on the microinfarcts came from only one time point, therefore our results are neither comprehensive nor do they reflect the complicated cascade of cellular changes. Further research is required to fully elucidate the dynamic changes following microinfarcts. This preliminary study has clearly demonstrated histopathologically the vascular, neuronal and glial alterations in all mice with the microinfarct model compared with those of vehicle controls, suggesting this is an effective model for microinfarcts. The limited number of animals focused our observations on the morphological changes. A larger study would be required for quantitative histological and statistical analysis. Finally, it should be noted that gender differences are well known in ischemic stroke (Ahnstedt et al., 2016). Our study used only male mice therefore female mice should be included in future studies using this model.

Vascular, neuronal, and glial alteration associated with microinfarcts

The fluorescent microbead ischemia model allowed us to analyze the multicellular alteration associated with multifocal microinfarcts at the early stage of ischemia. As with other types of ischemic stroke (del Zoppo, 2009, 2010; Barakat and Redzic, 2016; Iadecola, 2017; Freitas-Andrade et al., 2020), the breakdown of BBB and NVU is also thought to be the major features of the present model. Here, we demonstrated that the lodged microspheres greatly altered vascular integrity, resulting in vascular and neuronal injury, as well as astroglial and microglial activation in the insulted regions.

According to the expression of phalloidin and CD31 labeling, the smooth muscle cells and endothelial cells of the arterioles were obviously disrupted in the regions of microinfarcts. As the first-line defense to ischemia and hypoxia, endothelial cells play a critical role in maintaining the homeostasis of the brain under physiological conditions. However, endothelial dysfunction can increase BBB permeability and subsequently lead to lesioning of the brain parenchyma (Jiang et al., 2018; Mustapha et al., 2019).

In the present study, phalloidin was chosen as a suitable biomarker for labeling the cerebral arteries under both normal and pathological conditions. In contrast, CD31 labelled the capillaries in the regions of microinfarcts more strongly than those of controls. It is not yet clear whether this phenomenon is correlated with angiogenesis (Woodfin et al., 2007; Chazotte, 2010; Chistiakov et al., 2016; Alarcon-Martinez et al., 2018; Wang et al., 2020). In parallel to the vascular injury, neuronal degeneration was observed in focal regions of the microinfarcts six hours after the ischemia modelling. It reflects the neuronal and vascular vulnerability within the vascular territory-at-risk, indicating that vascular disorder with neurological consequences is an acute event for ischemic stroke (Rapp et al., 2000; del Zoppo, 2010; Kim et al., 2016).

Histological studies show that astrocytes are ideally positioned to conduct information in a bidirectional manner between neurons and blood vessels (Zonta et al., 2003; Takano et al., 2006; Mishra et al., 2016; Jiang et al., 2018). Our results clearly showed that astroglial activation was involved in the pathological alteration of blood vessels and neurons. Since activated astrocytes are mainly found in the region of microinfarcts and along the walls of blocked vessels, it implies that astrocytes could form a barrier to confine the spread of the lesion, although it may also result in the formation of glial scar to impede neurogenesis and neurological recovery (Jiang et al., 2018).

In contrast to astrocytes, microglia as the resident immune cells can also actively respond to the ischemic injury (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Tremblay et al., 2011). In the present model, although most of the activated microglia did not exhibit amoeboid morphology, they had enlarged cell bodies and short processes, an indication of activation against microinfarcts. However, the precise role of activated microglia during the ischemic stroke, whether detrimental or beneficial, is still poorly understood (Hu et al., 2012; Patel et al., 2013; Mallucci et al., 2015; Jiang et al., 2018).

Currently, our understanding of acute multicellular alteration to ischemic stroke is incomplete (Mustapha et al., 2019). As integral parts of the BBB or NVU, endothelial cells, neurons and glial cells are interconnected and interact, contributing to brain homeostasis under the physiological condition (Shih et al., 2006; Attwell et al., 2010; Andreone et al., 2015; Iadecola, 2017). However, this homeostasis is disturbed by ischemic stroke leading to rapid cellular changes (Prakash and Carmichael, 2015; Jiang et al., 2018; Freitas-Andrade et al., 2020). Although the present work only focuses on histopathological changes, we hope that this model will open a new technical window to understand the multicellular alteration associated with multifocal microinfarcts.

In summary, we have established an experimental model of ischemic stroke using fluorescent microspheres, which simulated multifocal microinfarcts causing multicellular changes. This model is a valid tool for investigating the underlying mechanisms of ischemic stroke and assessing its therapeutic intervention. Our histopathological findings also suggest that therapeutic intervention would be most effective for patients if carried out at the early stage of ischemic stroke.

Author contributions: Study conception and design: YS, MJY, WZB; model preparation: YS, MJY, YXS, GRW; histochemical staining and sample observation: YS, JW, JJC; data analysis and figure preparation: DSX, ILZ; manuscript drafting: YS, WZB. All authors approved the final manuscript.

Conflicts of interest: The authors declare no conflicts of interest.

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