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Short Communicaiton

Use of 2,3,5-triphenyltetrazolium chloride-stained brain tissues for immunofluorescence analyses after focal cerebral ischemia in rats

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ABSTRACT

The middle cerebral artery occlusion (MCAO) model in rodents has been widely used as model for studying brain ischemic stroke. TTC (2,3,5-triphenyltetrazolium chloride) staining in fresh tissues is used to evaluate the size of the infarct in MCAO model, and TTC-stained brain tissues are considered to be possible to bring a damage to the anatomical structure of neuronal cells and unsuitable for immunofluorescence analyses of cytology, and discarded after evaluation of infarct volume. Another group of models with in vivo fixation was required to the pathological or histological analyses of the infarct brains, which lead to double the numbers of animals in researches. However, some evidences indicate that if we properly optimized staining protocol, TTC-stained brain tissues might be suitable for cytological analyses. In this work, we have optimized the immunofluorescent staining methods of TTC-stained brain slices, and found that TTC-stained brain tissues are suitable for quantitative and qualitative analyses of microglia, astrocytes and neuroblasts, the morphology of theses cell were nearly identical to the in-vivo fixed models. Our optimized-protocol provide two advantages over traditional methods one of them is providing the precise the infarct region, which reduces the differences within groups, the other one is decreasing the total number of animals in research dramatically.

1. Introduction

Ischemic stroke is the leading cause of disability and mortality in aged-people, and there are not available medicine and/or therapies to cure these patients except for immediate recanalization by tissue plasminogen activator (tPA). However, the time window of tPA therapy is narrow, leading to almost 95% of patients have no chances for receiving immediate recanalization [1]. Therefore, experimental ischemic stroke models are essential to study the mechanism of cerebral ischemia and evaluate the effects of novel therapeutic interventions. The middle cerebral artery occlusion (MCAO) model in rodents has been widely used in basic research to study ischemic stroke. In MCAO model, the middle cerebral artery is occluded for a transient time (60 min to 120 min) or permanently, causing brain tissue ischemia and then resulting in enormous amount of cells death, called the infarct, which was considered irreversible before. However, accumulating papers showed that the infarct region can be repaired and rehabilitated by stimulating endogenous neurogenesis or cell transplantation [2,3]. The surrounding region, known as the penumbra, has bilateral consequences with time going on, recovering or progressing to the infarct [4]. The therapeutic options for the penumbra and infarct are different according to their different pathological conditions. Therefore, it is critical to distinguish the infarct and penumbra in the experiment studies.

TTC staining has been used extensively to visualize the penumbra and infarct for its character of facility, celerity, veracity and cost effectiveness. Generally, TTC reacts with the mitochondrial enzyme of living cells, forming the red compound (formazan), so the red region reveals the healthy/normal tissue. By contrast, the whiteness in the ischemic tissue shows the absence of living cells, which indicates the infarct region. This staining procedure clearly delineates the size of the infarct area and distinguishes the infarct core, the penumbra, and the healthy tissues [5]. Currently, TTC can be used to stain the brain slices in vitro [6] and intracardiac injection in vivo [7] to evaluate the ischemic size. However, TTC-stained brain tissues in vitro were discarded after measuring the infarct area in most studies. And whether TTC-stained brain tissues are suitable for the subsequent experiment to analyze molecular or cellular morphology changes is unclear. Previous studies have shown that TTC-staining process does not constrict quantitative gene and protein analyses in RT–PCR and Western blot [8]. Whether the TTC staining has effects on neuronal cell morphology has not been documented. Combing histological and pathological analyses with volumetric assessment is the golden standard for the assessment of

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drug therapy and management strategies in ischemic stroke researches [9]. The aim of the present study was to test whether TTC-stained brain tissues is suitable for immunofluorescence analyses to observe the cellular morphology changes in the infarct and penumbra regions.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (weighing 280–320 g, qualification number 2007000554501, lot number 2013-0118241) were purchased from the experimental animal's center of Silaike (shanghai, China). All procedures, including the MCAO model, were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Committee on Animal Research, Shanghai Medical College, Fudan University. Rats were housed in groups of 2, respectively, in standard cages under standard laboratory conditions (12-h light/12-h dark cycle, 22 ± 2 °C, food and water ad libitum). All rats were allowed to acclimatize to the housing facilities and were handled daily for at least 3 days. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.2. Transient middle cerebral artery occlusion (tMCAO)

Right MCAO was created as described before [10]. The animals were fasted overnight before surgery. Briefly, SD rats were anesthetized with 40 mg/kg ketamine (intraperitoneal injection) and allowed to breathe spontaneously. After the right common carotid artery was ligated, a 0.28 mm diameter monofilament (Beijing Sunbio Biotech Co. Ltd., Beijing, China) was inserted to a depth of 18–19 mm (beginning with the common carotid artery bifurcation) to block the origin of the right middle cerebral artery (MCA); after 2 h of MCAo thread, the monofilament was withdrawn to enable reperfusion for 72 h. Throughout the surgery, the body temperature was maintained at 37 ± 0.2 °C with a heating pad. After recovery from anesthesia, rats were placed back into their cages with free access to food and water.

2.3. Experimental procedure

SD rats were randomly divided into two groups (n = 8): TTC (2,3,5-triphenyltetrazolium chloride) staining group and Conventional treatment (Control) group for immunofluorescence. TTC group: three days after surgery, rats were anesthetized with 40 mg/kg ketamine and decapitated directly without any perfusion. Brains were removed quickly and frozen at 0 °C refrigerator for 10 min, then dissected into 6 sections (2-mm each) in coronal brain sections. And 2-mm brain slices were immersed for 10 min into 2% TTC (TTC, Sigma–Aldrich, St. Louis, MO) at room temperature (22–24 °C). And then TTC stained brain slices were postfixed in 4% icy paraformaldehyde for overnight. Afterwards, TTC stained and postfixed brain slices were scanned by a photo scanner. Control group: Rats were anesthetized with 40 mg/kg ketamine and intracardially perfused with 37 °C phosphate buffered (PBS)saline for 10 min followed by 4% icy paraformaldehyde in PBS for 30 min. Following perfusion, brains were dissected into 6 sections, and postfixed in 4% paraformaldehyde in PBS for 4 h. Both two groups subsequently dehydrated with 30% sucrose in 0.1 M PBS for 24–36 h at 4 °C. The brain slices in the two groups were embedded in Tissue-Tek OCT compound for 30 min at −20 °C and cut into coronal sections (40 μm thickness) on a freezing microtome (Leica CM 900; Leica, Nussloch, Germany)). Floating brain sections were preserved in the brain protection fluid (30% sucrose +30% glycol in PBS) to be used.

2.4. Immunofluorescence and confocal microscopy scanning

Brain sections were washed in 0.1 M PBS 15 min, and incubated with blocking solution (0.2% Triton X-100 and 10% goat serum in 0.1 M PBS) for 60 min at room temperature. Sections were then incubated with primary antibody in dilute solution (0.2% Triton X-100 and 5% goat serum in 0.1 M PBS) overnight at 4 °C. After rinsing with PBS for 5 × 10-min, sections were incubated with the secondary antibodies in dilute solution (5% goat serums in 0.1 M PBS) for 1 h in the ice box. The following antibodies and reagents were used in the protocol: mouse anti-GFAP (1:1000; abcam, ab10062); goat anti–Iba-1 (1:1000; abcam, ab5076); goat anti-DCX (1:250, Santa cruz). According to the primary antibodies recognized: goat anti-rat Alexa Fluor 488-conjugated IgG and goat anti-rabbit Alexa Flour 546-conjugated IgG; After rinsing with PBS for 5 × 10-min. Sections were then mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) to the glass slide. Immunolabeled brain slices were imaged with a confocal laser scanning microscope (Zeiss LSM700, Zeiss, Germany) and images were analyzed with a three-dimensional (3D) constructor (ImageJ software). Tissue images presented in the paper were maximum projections exported as TIFF files using Zen software and imported into Adobe Photoshop. Brightness and contrast of entire digital images were adjusted to optimize cellular detail. Identical adjustments were performed on all images meant for comparison.

2.5. Statistical analysis

For statistical analyses, SPSS 15.0 for Windows was used. All values were expressed as mean ± SEM and analyzed using Student's *t*-test for two groups. In all cases, Statistical significance of the differences was defined by $P < 0.05$.

3. Results

3.1. In vitro TTC-stained brain slices following experimental stroke in adult rats

In the tMACO model, a significant brain infarct region could appear at 90 min after surgery, and the infarct region gradually broadened up to 24 h, then following a status called plateau from 24 h to 7d after surgery. Our investigation was carried out at the time of middle of plateau. The brains were quickly removed from the skull on three days after surgery. Previous studies about the TTC staining method showed that the 2-mm-thick brain slices were immersed for 30 min in 2% TTC solution at 37 °C [6]. However, in the present study, the 2-mm-thick brain slices were immersed in 2% TTC solution for 10 min at room temperature (22 °C to 24 °C). The 2-mm brain slices were intensely colored with 2% TTC solution even if they were immersed for a short time at 22 °C to 24 °C (Fig. 1A). The brain slices showed the obvious difference between the red (viable) and white (infarct). More importantly, the 40 μm brain slices mounted on the glass slide were still light pink in the viable tissue and light white in the infarct, in spite of being washed for a long time (Fig. 1B), which can be early and quickly located the infarct and penumbra at the time of using microscope for subsequent cellular morphology observation.

3.2. Microglial morphology was not affected in the TTC group

Microglia, a phagocyte in brain, can be activated rapidly and migrate to the ischemic area in response to brain insults. Microglia have been widely studied in the researches for its role of secreting proinflammatory cytokines [11] and eliminating the dead tissue and cells [12] in the context of ischemic stroke. They play different roles depending on the different microenvironments: ramified (also known as the resting state), migratory (the state between resting and activated), and activated (activated amoeboid state) [13]. Based on these characteristics, we have evaluated the morphology of microglia. To identify microglia, we used immunofluorescent labeling with an antibody against Iba-1 (ionized calcium binding adaptor molecule 1), Iba-1 could express in the entire cells, and outline both the cell body and the