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Research paper

Green-channel autofluorescence imaging: A novel and sensitive technique to delineate infarcts

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HIGHLIGHTS

- Novel GCAF-imaging is a reliable alternative to TTC staining in stroke experiments.
- GCAF imaging is more sensitive in the detection of subtle ischemic brain damage.
- GCAF imaging may be useful for identifying the ischemic penumbra.

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ABSTRACT

Background: We have found that infarcted brain regions exhibit green channel autofluorescence (GCAF). Here, we compare ex vivo GCAF-imaging with 2,3,5-triphenylteterazolium chloride (TTC)-staining.
New method: C57BL/6 mice (n = 120) underwent GCAF-imaging after transient or permanent middle cerebral artery occlusion (tMCAO or pMCAO).
Comparison with existing methods: TTC-staining may not reflect subtle ischemic injury. TTC-stained tissues, when reused, are prone to processing artifacts related to prior TTC-staining. GCAF imaging requires little experimental manipulation of animals and brain tissues, and allows for more consistent measurements of infarct volume and reliable reuse of the fresh unstained tissues.
Results: Lesion volumes measured at 24-h after 1-h tMCAO by using GCAF-images were similar to those using TTC-staining: $87.6 \pm 13.6\,mm^3$ vs. $83.8 \pm 12.8\,mm^3$ in 1 mm-thick sections (n = 9 mice, 10 slices/mouse, $p = 0.88$; Pearson's $r = 0.91$, $p < 0.001$) and $75.1 \pm 7.6\,mm^3$ vs. $73.6 \pm 6.7\,mm^3$ in 2 mm-thick sections (n = 9 mice, 5 slices/mouse, $p = 0.99$; Pearson's $r = 0.87$, $p < 0.001$), respectively. In serial ex vivo imaging performed at 1, 2, 3, 6, 12, and 24-h after tMCAO, GCAF-imaging correlated well with TTC-staining at all time-points. In the pMCAO model however, the correlation was strong at later time-points (6–24-h); but at time points up to 3-h, GCAF-imaging was more sensitive than TTC-staining to detect ischemic areas, as verified by histology, where ischemic damage was observed in the GCAF-positive areas of the cerebral cortex and striatum, even in the face of normal TTC-staining.
Conclusion: GCAF-imaging is a reliable alternative to TTC-staining in the qualitative and quantitative assessments of focal brain ischemia, and more sensitive for detecting early ischemic damage in pMCAO.

1. Introduction

To measure the infarct size in animal models of ischemic stroke, researchers have long used 2,3,5-triphenyltetrazolium chloride (TTC) staining (Bederson et al., 1986; Isayama et al., 1991). Whitish TTC is enzymatically reduced to red 1,2,5-triphenylformazan in liv-

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ing tissues by the activity of dehydrogenases that are abundant in mitochondria (Bederson et al., 1986). Imaging contrast on ex vivo brain slices develops between whitish colors in necrotic areas devoid of dehydrogenases, while healthy brain regions retaining dehydrogenase activity becomes stained with a deep red color.

TTC staining, though it is the current standard stain for infarct, has many shortcomings: (a) In most cases, TTC-stained tissues cannot be reused for other experiments, (b) TTC staining may not reliably reflect subtle ischemic injury with partial damage of mitochondria (Liszczak et al., 1984), and (c) the results of TTC staining are dependent on the method of perfusion (brain in vivo or brain section in vitro) and the concentration of TTC solution used (Benedek et al., 2006).

Autofluorescence is the intrinsic fluorescence emitted from intact or damaged cells and tissues, based on their inherent fluorescent characteristics (Chung et al., 1997). It is different from the fluorescence induced by exogenous fluorescent markers that label cells and tissue structures (Monici, 2005). Recently, we and others have reported that ischemic injury in the brain induces green-channel autofluorescence (GCAF) (Barber et al., 2012; Kim et al., 2011, 2010), and postulated that this GCAF may be used for delineating infarcted brain areas ex vivo to overcome some of the limitations of TTC staining.

In this study, we investigate GCAF imaging in detail by directly comparing it with TTC staining and histology in two widely-used mouse models of focal cerebral ischemia: transient middle cerebral artery occlusion (tMCAO) and permanent MCAO (pMCAO) models.

2. Materials & methods

This study, comparing ischemia-related GCAF signal areas and TTC-unstained areas in both tMCAO and pMCAO models, was approved by the Preclinical Research Institute of Dongguk University Ilsan Hospital. All experimental procedures were performed in accordance with National Institutes of Health guidelines. Animal experiments and image analyses were performed by K.-H.J. and S.-K.L., who were blinded to study groups (i.e. either tMCAO or pMCAO, imaged at different time points), into which animals had been randomly allocated.

2.1. Mouse stroke models

Specific pathogen free (SPF) male C57BL/6 mice (n = 150), which are frequently used in stroke research, were purchased (Orient Bio Inc., Seongnam, Korea) and maintained in standard cages under a controlled environment of 23 °C and 50% humidity, with 12 h of light per 24 h period, in our semi-SPF facility. We induced tMCAO (1 h occlusion followed by reperfusion for up to 24 h) or pMCAO (0.5–24-h occlusion), respectively in 11–12-week-old mice, as previously reported with some modification, (Kim et al., 2011; Park et al., 2014) using a standardized silicon rubbed-coated 6.0 nylon monofilament (602356PK10, Doccol Corp., Sharon, MA, USA). Mice were anesthetized in the same semi-SPF operating room with 2% isoflurane using an inhalation mask. Cerebral blood flow (CBF) was monitored over the ipsilateral MCA using a laser Doppler flowmeter (LDF; Omegawave, Tokyo, Japan), and a homeothermic blanket (Panlab, Barcelona, Spain) was used to maintain body temperature at 36.5 °C.

The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed by performing a midline neck incision and dissection of the *peri*-vascular tissue. The ECA was ligated and the origin of superior thyroid artery was coagulated. The CCA was ligated using sterile 7.0 black silk. Following this, the ICA was separated from the adjacent vagus nerve and ligated. After making a puncture in the ECA proximal to which it was tied, the nylon thread was inserted via the created defect and directed toward the ICA. Immediately after loosening the ICA ligation, the nylon thread was advanced approximately 9 mm toward the MCA—ACA (anterior cerebral artery) bifurcation area. In each animal, LDF confirmed that CBF decreased to less than 30% of the baseline value. For the tMCAO model, the occlusion was continued for 1 h, after which the nylon thread was carefully removed, followed by the ligation of the ECA and untying of the ligated CCA to restore CBF (>50%). For the pMCAO model, the occlusion was maintained until the mice were euthanized.

Thirty mice were excluded because of *peri*-operative death (n = 19) or incomplete CBF reduction (n = 7) or restoration (n = 4). Thus, a total of 120 mice without adverse events were included in the analysis.

2.2. Ex vivo GCAF imaging vs. TTC staining and histology

In 18 mice using the tMCAO model, the infarct areas were assessed both by GCAF imaging and TTC staining at 24 h. These animals were ischemic for the 1 h of thread occlusion, but were re-perfused by thread withdrawal. Before returning a mouse in the original cage, we kept it warm (35 °C, 50% humidity) in a portable animal intensive care unit (Thermocare Industries, Paso Robles, CA, USA) for 1 h. At 24 h after reperfusion, animals were sacrificed by cervical dislocation, and the fresh unfixed brains were removed and cut with either 2 mm or 1 mm thickness (n = 9 per each group) using a brain matrix device (Jeung Do Bio & Plant Co., Seoul, Korea). Each brain slice was imaged using a charge-coupled device camera (CoolSnap-EZ, Roper Scientific, Tucson, AZ, USA; excitation/emission, 482/535 nm; 1000 ms acquisition) to detect ischemia-related autofluorescence, i.e. GCAF. A reference standard (a fluorescent plastic bar, 18 × 6 × 1.5 mm) was used in every GCAF imaging. A white light non-fluorescent reference image was also obtained. Following the GCAF imaging, the fresh brain slices were stained with 2% TTC solution (Amresco, Solon, OH, USA) for 20 min at room temperature in the dark to delineate infarcted areas. The TTC-stained slices were photographed using a camera (FineFix S9000, Fuji Photo Film Co., Tokyo, Japan).

In an additional 30 mice, after GCAF imaging, we obtained fresh brain tissues for microscopic GCAF imaging (Olympus-BX53, Olympus, Tokyo, Japan; excitation/emission, 470–495 nm/510–550 nm), hematoxylin and eosin (H&E) staining, immunohistochemical staining, Fluoro-Jade B staining, Nissl staining, and electron microscopy, as such investigations cannot be reliably performed on TTC-stained tissues. Except for the H&E staining (using paraffin-embedded sections) and electron microscopy study (see below), fresh brain tissues were embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Tokyo, Japan) and stored at −80 °C.

For immunohistochemical staining (n = 3), 10 μm-thick coronal brain sections were fixed with acetone at 20 °C for 10 min, air-dried for 30 min, and rinsed using PBS. The fixed tissues were blocked with 5% bovine serum albumin (BSA) for 30 min. Thereafter, the sections were incubated with rabbit anti-NeuN antibody (1:100, Chemicon, Darmstadt, Germany), rabbit anti-IBA-1 antibody (1:100, Waco, Osaka, Japan), or rabbit anti-GFAP antibody (1:100, Abcam, Cambridge, UK) in 3% BSA in PBS-T overnight at 4 °C. Then, they were washed twice in PBS-T solution for 5 min, followed by incubation for 1 h at room temperature using secondary antibodies (AlexaFluor 594, Invitrogen, Carlsbad, CA, USA).

For Fluoro-Jade B staining (tMCAO and pMCAO models at 3 and 24 h, n = 3 per each model and time-point), 10 μm-thick coronal brain sections were immersed in 0.06% potassium permanganate solution for 30 min with gentle shaking in the dark. Thereafter, the slides were rinsed in distilled water and immersed in 0.004% Fluoro-Jade B solution (Millipore, Darmstadt, Germany) for 30 min