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Effect of TTC Treatment on Immunohistochemical Quantification of Collagen IV in Rat Brains after Stroke

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Abstract

Although used extensively in stroke research, there is limited knowledge of how 2, 3, 5-triphenyltetrazolium chloride (TTC) - treated rat brain sections are altered and if they can be used for immunohistochemical quantification after staining with TTC. In the present study, we hypothesized that TTC treatment (TTC+) would not interfere with collagen IV immunohistochemical staining compared with non-TTC-treated (TTC-) brain slices. We further hypothesized that there would be no difference in autofluorescence or nonspecific secondary antibody fluorescence between TTC+ and TTC- brain slices. Coronal brain sections of male Wistar rats (n=5/group) were either treated with TTC or not after middle cerebral artery occlusion or sham surgery, and processed for immunohistochemical staining with mouse anti-collagen IV as the primary antibody, and goat anti-IgM as the secondary antibody. Four images were taken in the cerebral cortex of contralateral side of infarction in each brain slice using an Olympus BX50 fluorescence microscope, and average intensity of the entire image was quantified using Metamorph software. Compared with TTC- brain slices, TTC+ brain slices showed a significantly lower autofluorescence ($P < 0.05$), but was unchanged for nonspecific secondary antibody fluorescence. In addition, TTC+ brain slices had similar collagen IV staining intensity compared with TTC- brain slices. These results demonstrate that TTC+ brain slices are usable for immunohistochemical quantification.

Keywords

Triphenyltetrazolium chloride; Collagen IV; Immunohistochemical quantification; Infarction; Ischemic stroke

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Compliance with Ethical Standards

Ethical approval: All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont and complied with the National Institutes of Health guidelines for care and usage of laboratory animals. This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest: The authors declare that they have no conflict of interest.

Introduction

2, 3, 5-triphenyltetrazolium chloride (TTC) is ‘vital dye’ that can be reduced by the mitochondrial enzyme succinate dehydrogenase [1] to a fat soluble, light-sensitive compound (formazan) that turns normal tissue deep red [2]. TTC is considered a vital dye because damaged tissue cannot reduce TTC, thus leaving the area white and thereby delineating abnormal areas [2]. The use of TTC staining has met with increasing acceptance in stroke research because it is inexpensive and offers a rapid method for detection and quantification of experimental cerebral infarction. In stroke preclinical therapeutic studies, TTC staining is commonly used to demonstrate decreased infarct volume after treatment with a neuroprotective agent [3–6].

Although used extensively in stroke research, there is limited knowledge of how TTC-treated rat brain sections are altered and if they can be used for quantitative immunohistochemical detection after staining with TTC. Regardless, several studies have used TTC stained brain sections for immunohistochemical studies [7, 8] and quantification of proteins using fluorescence intensity [4, 9–12] without considering the potential effects of TTC on the immunohistochemical composite staining, including autofluorescence, nonspecific secondary antibody fluorescence, and primary antibody detection. Thus, we investigated whether TTC-treated rat brain sections were altered and if they could be used for quantitative immunohistochemical detection. To investigate this, we used collagen IV to determine if there was any difference in immunohistochemical quantification, and compared the fluorescence intensity between TTC-treated (TTC+) brain slices and non-TTC-treated (TTC–) brain slices that were taken from rats that had undergone middle cerebral artery occlusion (MCAO). Collagen IV quantification was chosen because it is a major component of basement membrane of brain blood vessels. As an extracellular matrix protein that provides support, its turnover and alteration during ischemia does not change as rapidly as other proteins such as glial fibrillary acidic protein (GFAP) and neuronal nuclei (NeuN). Thus, collagen IV is relatively stable allowing us to better determine the effect of TTC staining on its quantification using immunohistochemical methods. In addition, for brains that were taken from animals that had undergone MCAO or sham surgery, the effect of TTC on staining was measured on the contralateral side to the infarction to eliminate any influence of ischemia on the brain tissue.

In the present study, we hypothesized that TTC treatment would not interfere with collagen IV staining compared with TTC– brain slices. We further hypothesized that there would be no difference in autofluorescence or nonspecific secondary antibody (‘no primary antibody’) fluorescence between TTC+ and TTC– brain slices.

Materials and Methods

Animals

All experiments were conducted using 16–19-week- old male Wistar rats. Animals were housed in the Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Animals had access to food and water *ad libitum*

and followed a 12-h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont and complied with the National Institutes of Health guidelines for care and usage of laboratory animals.

Experimental Protocol

Brain sections for comparison of immunohistochemical staining between TTC+ and TTC– brains were taken from previous studies. For TTC+ brain sections, five male Wistar rats were subjected to MCAO using the filament technique, as previously described [13]. However, all imaging was performed in the contralateral side to the infarction. Rats were euthanized under isoflurane anesthesia and brains were removed immediately. Two mm coronal sections (4–6 mm posterior to bregma) of the cerebral cortex were taken and incubated in 2% TTC (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) at 37 °C for 30 min. For TTC– brain sections, three male Wistar rats that were subjected to MCAO and two sham male Wistar rats were euthanized using isoflurane anesthesia, brains were removed immediately. Similarly, 2 mm coronal sections of the cerebral cortex were taken for following experiments, and those sections were labeled as TTC–. All TTC+ and TTC– brain sections were fixed overnight in 3.7% formalin at 4 °C, then transferred to 0.1 M PBS and paraffin embedded.

Immunohistochemistry

Immunohistochemical staining for collagen IV (Sigma, St. Louis, MO) was performed using standard procedures for both TTC+ and TTC– groups. Briefly, brain sections were embedded in paraffin, cut into 5 µm slices on a Leica RM2145 paraffin microtome, and placed onto hydrophilic adhesive slides (IHC World, Ellicott City, MD). Slides were allowed to air dry overnight at room temperature and then baked for one hour at 60 °C. Following deparaffinization and rehydration, slides underwent antigen retrieval with DAKO Target Retrieval Solution, pH 6.0 in 50% glycerol at 100 °C for 15 min, then 90 °C for 1 min in a decloaking chamber (Biocare Medical, Concord, CA). Slides were sequentially treated with 10% normal goat serum with 0.1% Triton X-100 for 1 hour, and incubated overnight at 4 °C with mouse anti-Collagen IV primary antibody (1 µg/ml). Slides were then incubated with goat anti-mouse secondary antibody (4 µg/ml; Invitrogen, Carlsbad, CA) for 1 hour at room temperature. To assess differences in autofluorescence, some sections underwent the same procedures without primary and secondary antibody incubation. To assess differences in nonspecific binding of secondary antibody, some sections underwent the same procedures without adding primary antibody.

Imaging

All imaging was done on the contralateral side to occlusion. For each brain slice, four images were taken continuously in the cerebral cortex of the contralateral side of infarction using Olympus BX50 fluorescence microscope (Tokyo, Japan) at 20× magnification, at the same brain region in all animals. The four images per slice were averaged such that the n value was the number of animals in the group.

Intensity Quantification

Digital images were created when the optical image of the tissue sample formed by the microscope was recorded by the camera, using a two-dimensional grid of equally sized pixels. The pixels spatially sampled the images, such that each pixel represented a defined sized area in a specific location in the tissue sample. During the acquisition of the images, the photons that were detected at each pixel were converted to an intensity value that is correlated to, but not equal to, the number of detected photons. Therefore, in the fluorescence microscope, the intensity value of a pixel is related to the number of fluorophores present at the corresponding area in the tissue sample.

To quantify the fluorescence intensity of a protein after immunohistochemical staining, images were imported into Metamorph software (Molecular Devices, CA). Then the image was calibrated as every pixel was equal to a $0.368 \mu\text{m} \times 0.368 \mu\text{m}$ square. The camera had 12 bit outputs, thus, pixel intensity was ranged from 0 to 4095 (since $2^{12}=4096$) arbitrary units (AU) [14, 15]. To avoid unwanted nonspecific fluorescence that comes from the imaging system, which includes the excitation source, camera and external light source [16], a lower intensity threshold, 160 AU, was applied through the whole imaging quantification for all images. Pixels with an intensity value less than 160 AU were not quantified, and those with an intensity value greater than 160 AU were quantified. Next, a histogram of intensity value was generated for each pixel based on the number of photons detected. Then, average intensity was calculated per image by following the formula: average intensity = total intensity values in one image / total amount of pixels in one image. The percentage of threshold area was calculated per image by following the formula: threshold area (%) = amount of pixels with an intensity value greater than 160 AU in one image / amount of total pixels in one image. Then, the average intensity of four images were averaged and displayed for each animal. Similarly, the percentage of threshold area of four images were averaged and showed for each animal.

Statistical Analysis

Data are presented as means $\pm$ standard error of the mean (SEM). Unpaired Student's t-test was used to determine differences between TTC+ and TTC- groups, and N value was five for both TTC+ and TTC- groups. Differences were considered statistically significant at $P < 0.05$. All graphs were created using GraphPad Prism (La Jolla, CA).

Results

Effect of TTC on autofluorescence and nonspecific secondary antibody staining

Fig. 1 showed representative photomicrographs of brain slices, without primary and secondary antibody, previously stained with TTC compared to those that were not stained with TTC. Autofluorescence was detected in both TTC+ and TTC- brain slices. However, TTC+ brain slices had a lower autofluorescence background compared with TTC- slices. Metamorph quantification (Fig. 1B) showed a significantly increased average intensity in TTC- slices than TTC+ slices (216.9 ± 16.0 AU vs. 176.1 ± 0.7 AU; $P < 0.05$), demonstrating a higher autofluorescence in TTC- slices compared with TTC+ slices. In addition, at the same lower intensity threshold, TTC+ brain slices showed a significantly

decreased percentage of threshold area compared with TTC– brain slices (Fig. 1C, $24.3 \pm 2.4\%$ vs. $72.6 \pm 8.7\%$; $P < 0.001$), suggesting that TTC treatment quenched autofluorescence background.

Similar to autofluorescence, nonspecific secondary antibody staining, as determined by omitting the primary antibody, was detected in both TTC+ and TTC– brain slices (Fig. 2A). Fluorescence quantification (Fig. 2B) revealed that there was no significant difference of nonspecific secondary antibody fluorescence intensity between TTC+ and TTC– brain slices (242.7 ± 10.7 AU vs. 254.5 ± 10.7 AU; $P > 0.05$). Additionally, at the same lower intensity threshold, TTC+ brain slices showed a similar percentage of threshold area compared with TTC– brain slices (Fig. 2C, $90.0 \pm 2.8\%$ vs. $91.8 \pm 2.2\%$; $P > 0.05$). Thus, TTC treatment did not affect nonspecific fluorescence from secondary antibody.

Effect of TTC on Collagen IV Staining Intensity

Collagen IV staining was detected in both TTC+ and TTC– brain slices (Fig. 3A). To accurately and precisely quantify the staining intensity of collagen IV, the same lower intensity threshold was set to reduce noise, autofluorescence and nonspecific secondary antibody fluorescence. As shown in Fig. 3B, fluorescence quantification indicated that there was no significant difference in collagen IV staining intensity between TTC+ and TTC– brain slices (267.6 ± 15.9 AU vs. 270.2 ± 14.9 AU; $P > 0.05$). In addition, TTC+ brain slices revealed a similar percentage of threshold area compared with TTC– slices (Fig. 3C, $92.2 \pm 3.4\%$ vs. $91.8 \pm 2.1\%$; $P > 0.05$). Therefore, TTC treatment did not have a significant effect on collagen IV staining intensity compared with TTC– brain slices.

Discussion

The main findings of this study were that compared with TTC– brain slices, TTC+ brain slices had notably decreased autofluorescence and percentage of threshold area, but were unchanged for nonspecific secondary antibody fluorescence. Additionally, TTC+ brain slices had similar collagen IV staining intensity and percentage of threshold area compared with TTC– brain slices. From these results we conclude that brain sections that are processed by TTC staining are viable for immunohistochemical quantification of collagen IV protein. A previous study demonstrated that TTC+ brain tissues in stroke animal models can be used for quantitative gene and protein expression analyses using real-time polymerase chain reaction (RT-PCR) and Western blot (WB). However, for those methods, TTC+ brain tissue was immediately snap frozen after TTC staining, and formaldehyde fixation was avoided since it could interfere with RNA or protein analysis [17]. Nonetheless, less is known of how TTC-treated and formaldehyde-fixed brain sections are altered and if they can be used for protein immunohistochemical quantification after staining with TTC. Our results add to the study of Kramer, showing that in addition to RT-PCR and WB, TTC+ brain sections may be viable for the immunohistochemical quantification of protein.

Unlike the fluorescence induced by exogenous fluorescent markers that are used in the immunohistochemical detection, autofluorescence is the intrinsic fluorescence emitted from intact or damaged cells and tissues [18]. Prior studies have shown that lipo-pigments [19], aromatic amino acids [20] and extracellular matrix [21, 22] contribute to autofluorescence

emission. It is also notable that pyridinic and flavin coenzymes are important endogenous fluorophores primarily located in mitochondria, which are involved in ischemic tissue damage [23]. Our results provide new knowledge that compared with TTC– brain slices, TTC+ brain slices had significantly decreased autofluorescence and percentage of threshold area. Future studies are needed to elucidate the mechanisms of TTC-related differences of autofluorescence signal intensities. Regardless, decreased autofluorescence but preserved antibody signal may be beneficial as it increases the signal to noise ratio.

To perform accurate and precise immunohistochemical quantification, a lower intensity threshold (160 AU) was applied to reduce various sources of noises. Noise causes imprecision in measurements of pixel intensity values in quantitative fluorescence microscopy, the major types are Poisson noise, thermal noise and read noise. Poisson noise, also referred to as signal noise or photon noise, comes from the signal itself. Measurements of stochastic quantum events, such as numbers of photons, are fundamentally limited by Poisson statistics [24]. Thermal noise is caused by stochastic generation of thermal electrons within the detector, and is largely eliminated by cooling of the cameras. Read noise is generated by the amplifier circuitry used to measure the voltage at each pixel, and is usually the dominant source of noise in standard cameras designed for quantitative imaging [25].

There are a few limitations to this study. First, collagen IV was the only protein we investigated and therefore we cannot exclude the possibility that other proteins in the brain can also be studied after TTC exposure. Second, we did not study and therefore unable to provide information about the effects of TTC on protein modifications, e.g., phosphorylation and translocation. Third, we chose to study only the contralateral side to ischemia and are aware that there may be different reactions to TTC exposure in brain cells of the ipsilateral side. However, we chose to study the contralateral hemisphere based on two considerations. First, brain tissue from the ipsilateral side had reduced exposure to TTC because of inability to take up TTC in inviable cells. Second, we could not separate the impact of ischemia versus TTC on collagen IV immunohistochemistry if ipsilateral side of the brain was studied. However, our results suggest that the use of collagen IV immunohistochemical quantification in TTC+ brain tissue is viable. One important benefit of this approach is to reduce the amount of animal use if post-TTC immunohistochemical analysis of protein is desirable. While we did not investigate if other proteins were similarly viable (e.g., GFAP, NeuN), but our experimental approach provides an easy measurement to investigate if TTC staining affects these proteins. Furthermore, while using Metamorph software to quantify the protein intensity, different lower intensity thresholds should be applied for accurate immunohistochemical quantification.

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Fig.1. Effect of TTC on autofluorescence of brain sections

A. Representative photomicrographs (20×) of autofluorescence from cerebral cortex of contralateral side of infarct from rat brain slices with or without TTC staining. **B.** Scatter plot graph showing the average staining intensity of autofluorescence from rat brains with or without TTC staining. **C.** Scatter plot graph showing the percentage of threshold area from rat brains with or without TTC staining. Lower intensity threshold of data quantification was set at 160 AU for both TTC+ and TTC- brain slices. Scale bar is 50 μm. Data were analyzed by unpaired Student's t-test (n=5). Differences were considered significant at $P < 0.05$. * $P < 0.05$, ** $P < 0.01$

Fig. 2. Effect of TTC on nonspecific secondary antibody fluorescence of brain sections

A. Representative photomicrographs (20×) of nonspecific secondary antibody fluorescence from cerebral cortex of contralateral side of infarct from rat brain slices with or without TTC staining. **B:** Scatter plot graph showing the average staining intensity of nonspecific secondary antibody fluorescence from rat brain slices with or without TTC staining. **C:** Scatter plot graph showing the percentage of threshold area from rat brain slices stained with or without TTC. Lower intensity threshold of data quantification was set at 160 AU for both TTC+ and TTC- brain slices. Scale bar is 50 μm. Data were analyzed by unpaired Student's t-test (n=5). Differences were considered significant at $P < 0.05$. No significant differences were detected (ns)

Fig. 3. Effect of TTC on collagen IV staining intensity of brain sections

A. Representative photomicrographs (20×) of collagen IV from cerebral cortex of contralateral side of infarct from rat brain slices with or without TTC staining. **B.** Scatter plot graph showing the average intensity of collagen IV from rat brain slices with or without TTC staining. **C.** Scatter plot graph showing the percentage of threshold area from rat brain slices stained with or without TTC. Lower threshold intensity was set at 160 AU for both TTC+ and TTC- brain slices. Scale bar is 50 μm. Data were analyzed by unpaired Student's t-test (n=5). Differences were considered significant at $P < 0.05$. No significant differences were detected (ns)