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Mannitol-facilitated perfusion staining with 2, 3, 5-triphenyltetrazolium chloride (TTC) for detection of experimental cerebral infarction and biochemical analysis

Yu-Yo Sun¹, Dianer Yang¹, and Chia-Yi Kuan^{1,2}

¹Division of Developmental Biology, Division of Neurology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

Abstract

A simple method to quantify cerebral infarction has great value for mechanistic and therapeutic studies in experimental stroke research. Immersion staining of unfixed brain slices with 2,3,5-triphenyltetrazolium chloride (TTC) is a popular method to determine cerebral infarction in preclinical studies. However, it is often difficult to apply immersion TTC-labeling to severely injured or soft newborn brains in rodents. Here we report an *in-vivo* TTC perfusion-labeling method based on osmotic opening of blood-brain-barrier with mannitol-pretreatment. This new method delineates cortical infarction correlated with the boundary of morphological cell injury, differentiates the induction or subcellular redistribution of apoptosis-related factors between viable and damaged areas, and easily determines the size of cerebral infarction in both adult and newborn mice. Using this method, we confirmed that administration of lipopolysaccharide 72 h before hypoxia-ischemia increases the damage in neonatal mouse brains, in contrast to its effect of protective preconditioning in adults. These results demonstrate a fast and inexpensive method that simplifies the task of quantifying cerebral infarction in small or severely injured brains and assists biochemical analysis of experimental cerebral ischemia.

Keywords

2,3,5-triphenyltetrazolium chloride (TTC); blood-brain-barrier; lipopolysaccharide (LPS); hypoxia-ischemia; cerebral ischemia; penumbra

1. INTRODUCTION

Immersion staining of fresh brain slices with 2,3,5-triphenyltetrazolium chloride (TTC) is a simple and popular method for detecting infarction in experimental stroke models (Bederson et al., 1986). TTC, a colorless water-soluble dye, is reduced to a deep red, water-insoluble compound (formazan) predominantly in the mitochondria of living cells, hence distinguishing between viable and infarcted brain tissue after stroke. However, immersion TTC-staining has its limitations because it is often difficult to section unfixed, edematous brains after severe ischemic injury, especially in newborn rodents. While perfusion staining of rodent brains by intracardiac injection of TTC solution was reported, we have found this

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²Send correspondence to Chia-Yi Kuan (alex.kuan@cchmc.org).

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method to be very inefficient (see Results), and the previous TTC perfusion-labeling method was rarely adopted in the literature (Isayama et al., 1991). The lack of a reliable *in-vivo* TTC-labeling method has increased the workload of quantifying tissue damage in experimental stroke, and uncertainty in distinguishing the core-versus-peri infarct areas for biochemical analysis.

Interestingly, although intracardiac injection of TTC hardly labels the brain, it stains the heart efficiently and has been used widely to detect experimental myocardial infarction. This disparity suggests to us that the entry of TTC dye to central nervous system may be hindered by the blood-brain-barrier (BBB). To test this hypothesis, we performed TTC perfusion-labeling after osmotic BBB disruption with mannitol (Rapoport & Thompson, 1973; Rapoport, 2000), and found a greatly enhanced brain-staining capacity. Here we report optimization of the TTC perfusion-labeling method that demarcates infarction in both adult and newborn mouse brains. Further, we show that the *in-vivo* TTC-labeling method is compatible with biochemical analysis and distinguish between viable and infarcted tissue. Finally, using this method, we showed that pre-exposure to a bacterial endotoxin lipopolysaccharide (LPS) 72 h before hypoxia-ischemia amplifies brain damage in newborn mice, in contrast to its effect of protective preconditioning in adults (Tasaki et al., 1997; Rosenzweig et al., 2004; Eklind et al., 2005). Additional applications of this new vitality-detection method are also discussed.

2. MATERIALS AND METHODS

2.1. Animal surgery

For adult cerebral hypoxia-ischemia (HI), eight-to-twelve week-old male CD1 (Charles River, Wilmington, MA) and Thy1-YFP mice (Jackson Laboratories Stock no. 003782, Bar Harbor, ME;) were challenged by transient cerebral HI, performed as described previously with minor modifications (Adhami et al., 2006; Shereen et al., 2011). Briefly, animals were anesthetized by intraperitoneal (IP) injection of avertin, and the right common carotid artery was ligated by two releasable (Mule) knots of 4-0 silt suture. After carotid ligation, mice were infused with 7.5% O₂ balanced by 92.5% N₂ through a face-mask for 60 min. The body temperature of mice was maintained between 37.5° to 38.5° C by Digi-Sense Benchtop RTD connected with a heating lamp and rectal probe (EW-89000-10; Cole Parmer, Vernon Hills, IL). The knots on the common carotid artery were released at the end of hypoxic stress.

For lipopolysaccharide (LPS)-sensitized neonatal cerebral HI, LPS (0.3 mg/kg, Sigma) was IP-injected to 5-day-old CD1 mice at 72 h before cerebral HI, as previously described with minor modifications (Eklind et al., 2005). At postnatal day 8, mice were anesthetized by 2% isoflurane and subjected to permanent ligation of the right common carotid artery. Mouse pups recovered for 1.5 h and were then exposed to hypoxia in glass chambers containing 10% oxygen and 90% N₂ in a waterbath kept at 37° C. After hypoxic exposure, mice were returned to dams in the animal care facility. The *in-vivo* TTC-labeling procedure was performed at 48 h recovery. These animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. *In-vivo* TTC-labeling method

To disrupt BBB, mannitol (0.5 M~ 1.4M, Sigma) prepared in PBS at a temperature of 37⁰ C was IP-injected to animals (~0.1 ml/g body weight) for 5 to 180 min as indicated in the text. Mice were anesthetized with avertin and transcidentally perfused of PBS followed by 10 ml of 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma). At 10 min after transcordial TTC

perfusion, the brains of animals were removed and placed into 4% paraformaldehyde. Alternatively, the extracted brains can be incubated in warm phosphate-saline buffer (PBS) for 30 min to enhance TTC-staining. For biochemical analysis, brains were removed after TTC-perfusion and divided into the contralateral (the left cortex) and lesion sides (the right cortex) for protein extraction.

2.3. Quantification of brain infarction

The post-fixed brains after *in-vivo* TTC-labeling were sectioned into 0.8 mm thick coronal slices with Vibratome (Stoelting, Wood Dale, IL) and photographed for quantification as previously described (Yang et al., 2009). Briefly, digital images of 5 slices in each brain were analyzed using the NIH ImageJ 1.4 software. Brain damage was expressed as the ratio of the infarcted area (white area in the right side) to the area of the undamaged, contralateral hemisphere.

2.4. Histology and immunohistochemistry

The fixed brains after *in-vivo* TTC-labeling were transferred into graded 30% sucrose solution and frozen in O.C.T. compounds for sectioning at 50 μ m-thickness using a sliding microtome (SM2000R, Leica, Wetzlar, Germany). YFP was visualized with an Olympus epifluorescent microscope (BX-51). For immunohistochemistry, the following antibodies were used: mouse anti-HSP70 (SPA810; Stressgen, Victoria, Canada), rabbit anti-DARPP32 (A31656; Chemicon, Temecula, CA), and mouse anti-microtubule-associated protein 2 (MAP2, Sigma, St. Louis, MO). The immunoreactivity was detected sequentially by biotinylated secondary antibodies, the Vectastain ABC kit (Vector Lab, Burlingame, CA), and the diaminobenzidine tetrahydrochloride (DAB) reaction.

2.5. Cell fractionation and immunoblot analyses

The cortex from fresh brain tissue after *in-vivo* TTC-labeling was subjected to protein extraction or mitochondrial-cytosol fractionation, as described previously (Adhami et al., 2006). For total protein extraction, three part of cortex (contralateral side with red staining and the ipsilateral side with or without red staining) were separated and homogenized in TLB buffer [20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 25 mmol/L β -glycerophosphate, 25 mmol/L Na-pyrophosphate, 2 mmol/L EDTA, 1 mmol/L Na_3VO_4 , 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Sigma)]. For mitochondrial-cytosol fractionation, the tissue was homogenized in cold buffer (20 mmol/L HEPES, pH 7.4, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl_2 , 1mmol/L EDTA, 1 mmol/L EGTA, 0.7% protease inhibitor cocktail, 1 mmol/L Na_3VO_4). The extracted protein samples were processed for immunoblotting and visualized by HRP-reactive chemiluminescence reagents (Amersham Biosciences, Arlington Heights, IL). Primary antibodies were rabbit anti-cytochrome c (#4272; Cell Signaling, Danvers, MA), goat anti-AIF (#sc9416, Santa Cruz, Santa Cruz, CA), mouse anti-cytochrome oxidase subunit IV (#A21348; Molecular Probes, Invitrogen, Carlsbad, CA), mouse anti-HSP70, rabbit anti-Bcl-xL (#sc634, Santa Cruz), rabbit anti-caspase-3 (#9662; Cell Signaling) and mouse anti- β -actin (#A544, Sigma).

2.6. Reverse-transcription PCR

The RNA from fresh brain tissues after *in-vivo* TTC labeling was extracted using TRIzol reagent (Invitrogen) for RT-PCR analysis, as described previously (Sun et al., 2010). Briefly, total RNA was processed with the high-capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA) to transcript cDNAs. The semi-quantitative PCR of mice *Hsp70*, *Caspase-3*, *Bim-EL*, *Bcl-xL* and *Tspo* cDNAs were detected using the following primers, and the cDNA of a housekeeping gene β -actin was measured in parallel

as an internal control: *Hsp70*, 5'-AAGCAGACGCAGACCTTCAC-3' and 5'-AGATGACCTCCTGGCACTTG-3'; *Caspase-35*, 5'-CTATCTGGACAGTAGTTACAAAAT-3' and 5'-CAGTCAGAGCTCCGGCAGTAG-3'; *Bim-EL*, 5'-CTACCAGATCCCCACTTTTC-3' and 5'-ACCCTCCTTGTGTAAGTTTC-3'; *Bcl-xL*, 5'-AGGCAGGCGATGAGTTTGAA-3' and 5'-TGAAGCGCTCCTGGCCTTTC-3'; *Tspo*, 5'-ATGGGGTATGGCTCCTACATAGT-3' and 5'-CCACTGACAAGCAGAAGATCG-3'; and β -*actin*, 5'-GAAGCACTTGCGGTGCACGAT-3' and 5'-GAAGCACTTGCGGTGCACGAT-3'. Reaction products were separated by electrophoresis on a 2% agarose gel. Bands were visualized using an electrophoresis image analysis system (Eastman Kodak Co., Rochester, NY).

2.7 Matrix metalloproteinase (MMP) zymography

MMP-9 and MMP-2 zymogram was performed, as previously described (Yang et al., 2009). Briefly, MMP-9 and MMP-2 in brain extracts was pulled-down using the gelatin Sepharose™ 4B beads (GE Healthcare, Buckinghamshire, UK) and separated by electrophoresis in a polyacrylamide gel containing 0.15% gelatin. After electrophoresis, gels were washed twice with 2.5% Triton X-100 and incubated in reaction buffer (50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl₂) at 37° C overnight. The gel was stained with Coomassie blue and destained to reveal the protease activity. Shown are the inverted image of zymogram gels.

2.8. Statistical Analysis

Quantitative data of infarct area were expressed as means $\pm$ SEM and compared between HI plus LPS and HI groups using unpaired *t*-test. Statistical significance was assumed at $p < 0.05$.

3. RESULTS

3.1. Optimization of TTC perfusion-labeling by mannitol-induced BBB disruption

First, we compared the ability of intracardiac versus intra-carotid artery perfusion of 2% TTC solution to stain the brain (Isayama et al., 1991; Dettmers et al., 1994). Whereas intra-artery (IA) injection of TTC worked better than intracardiac perfusion, neither method provided strong staining of the brain, despite intense labeling of the liver (Fig. 1A) and heart (data not shown). In contrast, following intraperitoneal (IP) injection of 1.4 M mannitol (250 mg/g body weight) that allowed the tail vein-injected Evans blue dye to label brains (Fig. 1B), the IA-injected TTC dye also stained the brain intensely (Fig. 1A). These results showed that osmotic opening of BBB by mannitol greatly increases the efficiency of brain-staining by TTC perfusion-labeling (Rapoport, 2000; Brown et al., 2004; McCarty et al., 2009; Louboutin et al., 2010).

To optimize the new *in-vivo* TTC-labeling method, we examined the effects of dosage and timing of mannitol-pretreatment on the staining intensity by intracardiac perfusion of TTC. This experiment showed a dose-dependent enhancement of TTC-stain by mannitol-pretreatment (Fig. 1C). Based on these data, we chose 250 mg/g mannitol (equivalent to 0.1 ml/g body weight of 1.4 M mannitol) for adult mice, and 312 mg/g mannitol (0.125 ml/g of 1.4 M mannitol) for newborn mice in subsequent experiments. We found at, at this concentration, a window of 30 to 120 min mannitol pretreatment provided the most intense and homogeneous brain labeling by TTC. In less than 30 min mannitol-pretreatment, the mouse cerebral cortex was faintly stained. Longer than 120 min, mice became enfeebled and brain staining was weak or uneven (Fig. 1D). Hence, we selected an interval of 30 min between mannitol-pretreatment and TTC-perfusion in the rest of experiments in this report.

3.2. *In-vivo* TTC-labeling following experimental stroke in adult mice

Next we tested whether the new *in-vivo* TTC-labeling method can detect infarction after experimental stroke. To do so, 8–12 week-old male CD1 or Thy1-YFP mice were subjected to thrombotic stroke induced by unilateral cerebral hypoxia-ischemia (HI) (Adhami et al., 2006). Brains were collected 24 h later by mannitol-facilitated TTC-labeling followed by transcardiac perfusion of fixatives. The brains were removed from the skull only after cardiac perfusion of fixatives. This procedure revealed a large area devoid of TTC-staining on the carotid artery-occluded side of brain (asterisk in Fig. 2A). Since the *in-vivo* TTC-stained brain were post-fixed, they were easily sectioned into 0.8 mm-thick slices while retaining a clear contrast of red (viable) and white (presumably infarcted) color contrast (Fig. 2B).

To ensure that the lack of TTC-staining signifies cell injury, we sectioned the brain into 50 μ m-slices for immunocytochemistry. The HI-injured hemisphere showed an increase of heat shock protein 70 (HSP70) staining, accompanied by reduction of dopamine- and cyclic AMP-regulated phosphoprotein 32 (DARPP32, a striatal neuron marker) and microtubule-associated protein 2 (MAP2, a dendritic marker) staining (Fig. 2C-E). These results show that *in-vivo* TTC-labeling can be combined with immunocytochemistry analysis. Yet, because repeated washing in immunocytochemistry diminished the contrast of TTC-stain, it is difficult to determine whether the boundary of TTC-stain corresponds to the border of ischemic cell injury.

To overcome this obstacle, we subjected Thy1-YFP mice—in which, a subset of cortical and hippocampal neurons express the yellow fluorescence protein (YFP)—to cerebral HI and *in-vivo* TTC-labeling, followed by direct visualization of neurons in 50 μ m slices. This analysis showed that neurons in the region devoid of TTC-staining (asterisk in Fig. 2F; the rectangles were magnified in Fig. 2G, H) either disappeared or showed severe destruction. This pattern suggests that the boundary of *in-vivo* TTC-labeling corresponds to morphological cell injury after experimental stroke.

3.3. *In-vivo* TTC-labeling is compatible with biochemical analysis of experimental stroke

While the toxicology properties of TTC have not been thoroughly investigated according to the manufacturer's material safety data sheet (Sigma, St. Louis, MO), we noticed that systemic administration of 2% TTC invariably kills mice rapidly, even without any experimental stroke challenge. This observation raised concerns whether the *in-vivo* TTC-labeling method will alter the cell death signaling responses to stroke.

To examine this issue, we first performed TTC perfusion-labeling in unchallenged (UN) or HI-injured mice at 16 h recovery, and collected the brains for mRNA and the mitochondrial protein distribution analysis (Fig. 3A-D; n=3–5 for each). RT-PCR analysis showed increase of the transcripts encoding *Hsp70*, *Caspase-3*, (pro-apoptotic) *Bim-EL*, and *18-kDa translocator protein (TSPO)*, a neuroinflammation marker (Martin et al., 2010) in TTC-negative tissue, where reduction of the (anti-apoptotic) *Bcl-xL* transcripts was evident (Fig. 3A, B). Cell-fractionation followed by immunoblot analysis showed that *in-vivo* TTC-labeling *per se* caused little or no leakage of cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria. In contrast, HI-injured and TTC-negative tissues showed conspicuous redistribution of cytochrome c and AIF from the mitochondria to cytosol (Fig. 3C, D). Of note, AIF was shortened from 62 kDa in the mitochondria to 57 kDa in the cytosol in TTC-negative regions, similar to a previous report using fresh brain sample after focal ischemia for analysis (Cao et al., 2007).

To further examine the suitability of *in-vivo* TTC-labeling for biochemical analysis, we compared the protein levels of HSP70, Bcl-xL, and pro-Caspase-3 in the unchallenged

(UN), the contralateral hemisphere (L), and peri-infarct (R/TTC+) or core regions (R/TTC-) at 16 h post-HI (Fig. 3E, F; n=3). We found that the peri-infarct tissue contained a higher level of HSP70 than the core, in accord with previous reports (Kinouchi et al., 1993). The reduction of Bcl-xL and pro-Caspase-3 protein levels was also evident in the HI-injured hemisphere.

Finally, we tested whether the *in-vivo* TTC-labeling procedure interfered with gelatin zymography that detects cerebral ischemia-induced matrix metalloproteinase-9 (MMP-9) and -2 (MMP-2) activities (Rosenberg et al., 1998; Park et al., 2009). This experiment showed that HI induced significant MMP-9 and MMP-2 activity only on the carotid artery-ligated hemisphere, in samples extracted from fresh (no TTC) or TTC-stained brains (Fig. 3G, H, n=4). Together, these results suggest that the *in-vivo* TTC-labeling procedure is compatible with biochemical analysis of cerebral ischemia.

3.4. *In-vivo* TTC-labeling of neonatal hypoxic-ischemic brain injury in mice

Next, we tested whether the *in-vivo* TTC-labeling procedure can be used to quantify HI-induced injury in P10 mouse pups, whose small brain sizes have excluded the use of immersion TTC-labeling in the past. Further, we used *in-vivo* TTC-labeling to examine the reported injury-sensitization effect by lipopolysaccharide (LPS) pre-exposure at 72 h before HI in rodent pups (Eklind et al., 2005).

We found that the *in-vivo* TTC-labeling method (0.125 ml/g body weight of 1.4M mannitol was used in mouse pups) readily stains the brain of P10 mice that were subjected to a low-dose (0.3 mg/kg) LPS pre-exposure at P5 and HI-insult at P8 (unilateral carotid ligation and 20 min 10% oxygen) (Fig. 4A, B). Simple inspection of TTC-stained brains suggested greater damage in the LPS72h/HI group. When the TTC-stained brains were post-fixed and sliced for quantification, we found a three-fold increase of infarction by LPS pretreatment (Fig. 4C; 8.4% in the HI group [n=10] compared to 26.8% in the LPS72h/HI group [n=14]; asterisk: $p < 0.05$ by *t*-test). These results indicated that the new *in-vivo* TTC-labeling method can be used to quantify infarction in neonatal stroke. Further, our results support the reported injury-amplification effect by 72 h LPS pre-exposure in newborn rodents, in contrast to its protective preconditioning effect in cerebral ischemia in adults (Tasaki et al., 1997; Rosenzweig et al., 2004; Eklind et al., 2005).

4. DISCUSSION

4.1. Osmotic opening of BBB facilitates TTC perfusion-labeling

The ability to quickly determine the location and extent of infarction after experimental cerebral ischemia is important for the investigation of underlying mechanisms and assessment of new interventions. While immersion TTC-staining is a very useful method to quantify cerebral infarction, it is less applicable to severely injured, edematous brains or smaller newborn brains in rodents. The alternative methods, including comparison of neural injury scores or quantification of the area devoid of Nissl or MAP2 staining, are all either semi-quantitative or labor-intensive. Hence, inspired by recent reports of mannitol-facilitated CNS-entry of viral vectors (McCarty et al., 2009; Louboutin et al., 2010), we adapted this approach to develop a TTC perfusion-labeling method. The new *in-vivo* TTC-labeling method is simple, fast, and reliable. Furthermore, it is applicable in both adult and neonatal brains after experimental stroke, delineates the boundary of ischemic cell injury, and supports biochemical analysis of experimental stroke.

Previous studies have established that the mechanism of mannitol-induced BBB opening is through vasodilatation and the shrinkage of cerebrovascular endothelial cells that collectively widen inter-endothelial tight junctions (Rapoport, 2000; Brown et al., 2004).

Our observation of mannitol-pretreatment facilitating the CNS-entry of Evan blue dye suggests that TTC, a small-molecule chemical, is also bound by larger proteins in the blood and thus unable to cross BBB. Of note, the TTC perfusion-labeling method requires a three-fold higher dose of mannitol in IP administration than those used for introducing virus to the CNS. Accordingly, while the window of mannitol-facilitated viral entry to the brain is less than 20 min (McCarty et al., 2009), the TTC perfusion-labeling method provides a longer duration of (30 to approximately 120 min following mannitol pretreatment) of brain staining. The increased temporal window in mannitol-facilitated TTC-labeling is useful when this method is applied to a group of animals at the same time.

4.2 The limitation of *in-vivo* TTC-labeling method

The new *in-vivo* TTC perfusion-labeling method, however, shall be considered a terminal procedure for two reasons. First, because this method uses a three-fold greater dose of mannitol pretreatment than that required for virus entry in the CNS, the osmotic opening of BBB may become irreversible. For example, we noticed substantial brain staining by TTC-labeling even at 3 h after mannitol administration (Figure 1). Further, due to the distress associated with injection with high-osmolarity solutions, the tolerable dose of mannitol for severely injured animals may require adjustment depending on the experimental conditions.

Secondly, while the previous reports of *in-vivo* TTC labeling did not describe any toxicity of this compound, and the manufacturer's material safety data sheet (MSDS) only stated that the toxicology of TTC has not been thoroughly investigated, we have found it to be highly toxic to animals in systematical administration. Even in unchallenged healthy mice, intra-artery or intracardial injection of 2% TTC invariably led to the death of animals immediately. TTC is a cell-permeable colorless chemical that is reduced to a water-insoluble deep-red formazon compound predominantly in the mitochondria of living cells, thus distinguishing between viable (red) and non-viable (white) tissue. However, TTC is also a powerful inhibitor of aerobic oxidation in the mitochondria (Sato and Sato, 1965). Hence, TTC perfusion-labeling is a terminal procedure and animals shall reach deep anesthesia prior to intracardiac or intravenous infusion of TTC solution.

4.3. *In-vivo* TTC-labeling supports biochemical analysis of the ischemic penumbra area

So what are the unique applications by the *in-vivo* TTC-labeling method in experimental stroke research? First, this method is particularly useful for quantifying infarction in the severely injured, edematous brain or in research of neonatal cerebral HI, because it allows post-fixation to produce thin brain slices with clear red (viable)-white (infarct) color contrast. Secondly, by clearly demarcating live-versus-dead tissue, it can help decipher the signaling events inside the penumbra area, which is of particular interest for the development of neuron-salvaging strategies (Sharp et al., 2000).

In this regard, a recent study has shown that immersion TTC-staining is compatible with mRNA and protein analysis (Kramer et al., 2010). Our study further indicates that biochemical analysis after *in-vivo* TTC-labeling recapitulates many hallmarks of apoptosis in experimental stroke, including the mitochondria leakage of cytochrome c and cleavage of AIF, as well as, a greater induction of HSP70 in the peri-infarct area (Figure 3) (Kinouchi et al., 1993; Cao et al., 2007). With its ability to demarcate the boundary of infarction precisely, *in-vivo* TTC-labeling can assist genomics-proteomics analysis of the ischemic penumbra area.

4.4. Age-differential effects of LPS-pretreatment on experimental stroke

Finally, to test the utility of *in-vivo* TTC-labeling method in newborn mice, we confirmed the report that 72 h LPS pretreatment increases HI brain damage in neonates, in contrast to the protective preconditioning effect of 24 h LPS pretreatment in neonates (Eklind et al., 2005; Lin et al., 2009) or by 24-to-96 h LPS pretreatment in adults (Tasaki et al., 1997; Rosenzweig et al., 2004). Verification of this finding is important, because although IP injection of a low-dose LPS (0.3 mg/kg) at 4–6 h before HI is a popular paradigm of infection-sensitized neonatal brain injury via the innate immunity (Lehnardt et al., 2003), some investigators remain critical of the acute LPS-pretreatment paradigm, complaining that it does not mimic chronic inflammation in intrauterine infection. By confirming the previous report of intensified HI brain injury by 72 h LPS-pretreatment, our results suggest an additional model to study the mechanisms and therapies of infection-sensitized HI brain injury in neonates. However, whether the innate immunity and neonatal inflammatory response remain activated at 72 h after LPS pretreatment remains unclear and warrants further investigation.

5. CONCLUSION

In conclusion, our results demonstrated a mannitol-facilitated TTC perfusion-labeling method that is a useful alternative to the *ex-vivo* immersion-labeling method. The *in-vivo* TTC-labeling method is simple, inexpensive, and particularly useful for measuring injury in severely damaged or small newborn brains. This new vitality-detection method not only simplifies the task of quantifying infarction, but also supports biochemical analysis of the core- and peri-infarct areas in experimental stroke.

Highlights

- Osmotic opening of blood-brain-barrier allows *in-vivo* TTC labeling of the brain.
- *in-vivo* TTC-labeling quantifies infarction in badly injured or small newborn brains.
- *in-vivo* TTC-labeling supports biochemical analysis of the ischemic penumbra area.

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Figure 1.

Osmotic opening of BBB by mannitol facilitates perfusion TTC-labeling of brains. **(A)** Comparison of the staining of brains and livers at 10 min after (from left to right) intracardiac perfusion of saline, intracardiac perfusion of 2% TTC, intra-carotid artery (IA) injection of TTC, and IA-injection of TTC following intraperitoneal (IP) administration of mannitol. Noted that IA perfusion of TTC with mannitol pretreatment produced deep red-staining in brains. **(B)** Mannitol pretreatment also allow the tail vein-injected Evans blue to cross the BBB and stain the brain. **(C)** Increasing concentration of mannitol pretreatment produced stronger staining of the brain by intracardiac perfusion of 2% TTC. The concentrations of mannitol (90, 180 and 250 mg/ g body weight) are indicated. **(D)** Time-dependent effect of mannitol pretreatment (250 mg/g, equivalent to 0.1 ml/g of 1.4M mannitol solution, IP) on TTC-stained whole brain and sectioned 0.8 mm slices. Noted that the best temporal window for mannitol pretreatment at this concentration is 30 to 120 min. UN: unchallenged. At least 3 brains were examined for each experimental condition with identical results.

Figure 2.

Morphological applications of *in-vivo* TTC-labeling in experimental cerebral ischemia in adult mice. The animal model of unilateral stroke was performed as described (Adhami et al., 2006). **(A-B)** The *in-vivo* labeling method detected a region devoid of TTC staining on the carotid artery-ligated hemisphere that was readily discernible either on whole-brain **(A)** or 0.8 mm slices **(B)**. **(C-E)** Immunostaining on cryostat sections of *in-vivo* TTC-stained brains (50 µm) showed specific increase of HSP70, as well as, decrease of DARPP32 and MAP2 labeling on the HI-challenged hemisphere (indicated by asterisks), suggesting that perfusion TTC-staining does not constrict subsequent immunocytochemistry analysis. **(F-H)** 50 µm brain slices of HI injured Thy1-YFP mice showed an area of reduced TTC-staining (asterisk). Direct visualization of YFP expression in regions with TTC-staining **(G)** contained numerous neurons in the cerebral cortex (Ctx) and neurites in the corpus callosum (CC) or the striatum (St). In contrast, very few YFP+ neurons or neurites were present in regions with TTC-staining **(H)**. Shown are typical results in $n > 5$ for each experiment in A-H.

Figure 3.

Biochemical applications of *in-vivo* TTC-labeling in experimental adult HI brain injury models. (A, B) RT-PCR analysis and quantification of the mRNA extracted from TTC-stained unchallenged (UN) and unilateral HI-injured brains (L: left and contralateral hemisphere to HI; HI(R): Right/HI-injured with TTC stain-negative region). Note the increase of *Hsp70*, *Caspase-3*, *Bim-EL* and *Tspo* mRNA, as well as, decrease of *Bcl-xL* mRNA in the core region [HI(R)]. Data represent means $\pm$ S.E.M in three sets of samples. Fold of indicated mRNA expressions are shown to compare with L group. * $p < 0.05$ by t-test. (C, D) Cell-fractionation and immunoblot analysis after *in-vivo* TTC-labeling showed release of cytochrome c and AIF from mitochondria (M) to the cytosol (Cyto) in the core of

infarction [HI(R)]. Note the cleavage of AIF from 62 KDa in mitochondria to 57 KDa in the cytosol. The purity of mitochondria-cytosol fractionation was shown by immunoblotting against cytochrome oxidase subunit IV (COX IV). Fold of protein expressions are shown to compare with L group in three sets of samples. $*p<0.05$; $**p<0.01$ by *t*-test. **(E, F)** Western blot analysis of total protein lysates showed the induction of HSP70, as well as, reduction of Bcl-xL and pro-Caspase-3 in per-infarct (HI-R/TTC+) and core (HI-R/TTC-) regions. Shown are typical results in three sets of samples. $*p<0.05$; $**p<0.01$ by *t*-test. **(G, H)** Gelatin zymography showed the induction of MMP-9 and MMP-2 in non-TTC- stained (*lane 3*) and TTC-stained (*lane 5*) HI(R). Shown are the fold of MMP induction in the injured (R) hemisphere compared to the contralateral hemisphere (L) in four sets of samples. $**p<0.01$; $***p<0.001$ compared to the contralateral side by *t*-test.

Figure 4.

Detection of brain infarction by *in-vivo* TTC-labeling in experimental neonatal HI brain injury models, performed as described (Yang et al., 2009). (**A**, **B**) Inspection of perfusion TTC-stained neonatal mouse brains showed that 72 h LPS pretreatment (0.3 mg/kg IP at P5) enlarged the HI-induced cerebral injury (unilateral carotid ligation followed by 20 min hypoxia with 10% O₂). (**C**) Quantifications showed a significant increase of the TTC-negative, infarcted area by 72 h LPS pretreatment from 8.4% of hemisphere in the HI group (n=10) to 26.8% in the LPS72h/HI group (n=14) (Shown are the mean and standard error of each treatment group. *: $p < 0.05$ by *t*-test).