Metabolic Quantification of Lesion Volume following Experimental Traumatic Brain Injury in the Rat

BRIAN R. PERRI,1 DOUGLAS H. SMITH,1 HISAYUKI MURAI,1 GRANT SINSON,1 KATHRYN E. SAATMAN,1 RAMESH RAGHUPATHI,1 RAYMOND T. BARTUS,2 and TRACY K. McINTOSH1

ABSTRACT

A reliable and rapid method for quantifying lesion volume following traumatic brain injury (TBI) has vast potential in brain injury research. Staining with 2,3,5-triphenyltetrazolium chloride (TTC) provides for demarcation of damaged or infarcted tissue from normal, viable cerebral tissue, in which a red formazan product is formed by reduction during cellular respiration of mitochondrial dehydrogenase enzymes. The present study evaluated the use of TTC staining to quantify the cortical lesion volume in rats undergoing fluid-percussion (FP) brain injury. Male Sprague–Dawley rats (350–450 g, n = 27) were anesthetized (sodium pentobarbital, 60 mg/kg, ip) and subjected to lateral FP brain injury of mild (1.1–1.3 atm, n = 5), moderate (2.0–2.3 atm, n = 9), or high (2.4–2.6 atm, n = 8) severity, while sham (noninjured) animals (n = 5) were anesthetized and surgically prepared without injury. Forty-eight hours after injury animals were sacrificed, brains were stained with TTC, and lesion volumes were calculated. A highly significant correlation was found between cerebral cortical lesion volume (mm³) and severity of brain injury (r = 0.85; p < 0.0001). The mean (± SD) lesion volumes were 12.1 (± 4.5) mm³ following mild injury, 33.8 (± 8.6) mm³ following moderate injury, and 45.1 (± 14.0) mm³ following severe injury. A significant difference was observed between all injury groups using a t test with Bonferroni correction (p < 0.05). These results suggest that the TTC staining technique is a useful, rapid, and reproducible method for quantification of lesion volume following lateral FP brain injury.

Key words: brain injury, histopathology, lateral fluid-percussion, lesion volume, metabolism, TTC

INTRODUCTION

In the acute phase following traumatic brain injury (TBI), endogenous autodestructive events (e.g., oxygen free radical and excitotoxic amino acid release) are initiated that contribute to secondary or delayed tissue damage (Pitts and McIntosh, 1990; McIntosh, 1994). Parasagittal fluid-percussion (FP) brain injury in the rat has been shown to produce vascular disruption, blood flow alterations, breakdown of the blood–brain barrier (BBB), extravasation of potentially harmful blood-borne products, regional cerebral edema, intraparenchymal hemorrhage, neural tissue shearing, and membrane disruption, all of which contribute to the formation of a focal lesion within the (ipsilateral) cortex (Cortez et al., 1989; Yamakami and McIntosh, 1989, 1991; Soares et

1Division of Neurosurgery, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
2Alkermes, Inc., 64 Sidney Street, Cambridge, Massachusetts 02139.
CURRENT THERAPEUTIC INTERVENTION strategies target the ensuing secondary injury mechanisms that contribute to posttraumatic tissue damage (Faden, 1993; McIntosh, 1994). To quantify regional neuronal loss and cellular damage in experimental models of brain trauma, investigators have relied upon often cumbersome conventional histopathological methods.

2,3,5-Triphenyltetrazolium chloride (TTC) was originally developed for use in botanical studies as a marker for seed viability, and has since been incorporated into biomedical research in models of myocardial, hepatic, renal, and skeletal muscle ischemia. The utility of TTC is based upon its reduction by mitochondrial enzymes such as succinic dehydrogenase, a red colored formazan byproduct. While viable tissue stains red, nonrespiring tissue is incapable of reducing TTC and remains white. Recently, staining with TTC has been utilized for quantification of lesion volume in models of focal cerebral ischemia (Liszczak et al., 1984; Bederson et al., 1986; Park et al., 1988; Cole et al., 1990; Isayama et al., 1991; Saeed et al., 1993). Furthermore, TTC staining has been used to evaluate therapeutic interventions aimed at preserving neural tissue following experimental focal cerebral ischemia (Bose et al., 1988; Marinov et al., 1991; Menezawas et al., 1992; Clark et al., 1993; Cole et al., 1993; Kiyota et al., 1993; Ranjan et al., 1993; Bartus et al., 1994a,b; Elger et al., 1994; Heinel et al., 1994).

In models of focal cerebral ischemia, pharmacologic efficacy is commonly defined by neuroprotective effects that are typically demonstrated by decreases in lesion volume. However, surprisingly few studies in models of trauma have utilized histopathologic analysis to evaluate therapeutic effects, relying normally on functional, neurochemical, or physiologic endpoints (McIntosh et al., 1989; Yamakami and McIntosh, 1989; Smith et al., 1991, 1993; Yoshino et al., 1991; Hovda et al., 1992; Soares et al., 1992; Vink, 1994). Therefore, the availability of an inexpensive, rapid, and reliable method for quantifying lesion volume following TBI would have widespread application in neurotrauma research. In the present study we evaluated a modified technique using TTC for rapid detection and quantification of lesion volume in rats subjected to lateral FP brain injury.

MATERIALS AND METHODS

Animal Surgery and Injury

The surgical preparation, injury procedure, and FP device have previously been described in detail (McIntosh et al., 1989). Briefly, male Sprague-Dawley rats (350-450 g, n = 27) were anesthetized (60 mg/kg sodium pentobarbital ip). Rats were placed in a stereotaxic frame and a left parietal craniotomy, 5.0 mm in diameter, was made midway between lambda and bregma and 2.5 mm lateral to the sagittal suture. The dura was left intact. A stainless-steel screw was secured to the skull over the left frontal cortex as an anchor for dental cement. A female Luer-Lok connector (3.5 mm inner diameter) was fixed securely into the craniotomy with gel super glue and dental cement. Ninety minutes following pentobarbital administration, anesthetized animals were attached to the injury device via the Luer-Lok connector and subjected to parasagittal FP brain injury of mild (1.1-1.3 atm, n = 5), moderate (2.0-2.3 atm, n = 9), or high (2.4-2.6 atm, n = 8) severity. Control animals (n = 5) were anesthetized and surgically prepared, but were not brain injured. All animals were allowed to recover without ventilatory or respiratory support and normothermia (37°C) was maintained using heating pads.

TTC Staining Procedure

Forty-eight hours after FP brain injury, rats were reanesthetized with sodium pentobarbital (200 mg/kg, ip), decapitated, and their brains rapidly removed. Although the lesion cavity is typically more fully developed by 1 week postinjury (Soares et al., 1995), we chose 48 h for TTC analysis to determine whether this technique could be used for more rapid assessment of lesion size and to evaluate whether the extent of the lesion at 48 h was reflective of the ultimate cavity that forms by 1 week. The rationale for selecting 48 h postinjury for lesion volume analysis was also based on results from investigations in models of cerebral ischemia using TTC staining (Liszczak et al., 1984). Brains were secured with agarose onto a chilled stage, and then refrigerated for 2 min. Each brain was cut into 1-mm coronal sections using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. LTD., Gomshall, Surrey, England). The fresh brain sections were separated in a bath of 0.2 M phosphate buffer at 37°C. All agarose was removed and the phosphate buffer was drained from the dish. The tissue sections were immersed in 2% TTC (Sigma Chemical Co., St. Louis, MO), prepared in 0.2 M phosphate buffer, and incubated at 37°C for 7 min. Sections were covered by glass microscope slides to ensure even staining, and light protected by covering the dish with foil. After staining, the tissue sections were washed twice with 0.2 M phosphate buffer and stored in 10% neutral buffered formalin.

Tissue Section Analysis

All imaging and lesion volume analysis was performed by an independent investigator blinded to the
presence (or severity) of the injury. Digitized, monochrome images of the rostral side of each section (4–10 sections per animal, depending on the extent of the injury) were captured and stored using a Dage-MTI CCD72 color video camera and M1-MCID imaging system software (Imaging Research, Brock University, St. Catherines, Ontario, Canada). For each section, lesion area was determined by enlarging the image and utilizing a standardized imaging scheme that allows for demarcation between red tissue (normal—suggesting high mitochondrial activity), pink tissue (damaged—suggesting reduced mitochondrial respiration), and white tissue (minimal or no mitochondrial activity). The white and pink tissue regions were defined as the area of lesion. In each of the 1 mm coronal brain sections presenting with white and/or pink tissue, the lesion area was measured and single section lesion volume was calculated (area × 1 mm thickness). This single section lesion volume was added to that of all other serial sections with lesions, resulting in a total (rostral-caudal) lesion volume, expressed in mm³.

The lesion volumes for the three injury groups were compared using a two-way analysis of variance (ANOVA). The mean values for each injury group were then compared by t tests with a Bonferroni correction for multiple comparisons. Linear regression analysis was also performed to test for significance in the correlation between injury level and lesion volume.

Histology

Representative samples of immersion-fixed, TTC-stained 1-mm slices were cut into 60-μm sections using a vibratome. These sections were mounted on glass slides and washed in ethyl alcohol to remove the TTC stain. The sections were then stained with hematoxylin and eosin (H&E). Specimens were examined using light microscopy for the presence of macrophages or glial cells that could react with the TTC, causing an underestimation of lesion volume.

For comparison of TTC-stained sections with conventionally stained sections demonstrating an evolved lesion, an additional four animals were subjected to FP brain injury at high severity (2.4–2.6 atm). One week following injury, these animals received an overdose of pentobarbital (200 mg/kg) and were transcardially perfused with 4% paraformaldehyde. The brains were removed, embedded in paraffin, cut in 6-μm sections, and stained with the Nissl-like stain, toluidine blue. The general size and location of the lesion at 1 week postinjury, demonstrated by toluidine blue staining, was then compared to the TTC-stained sections of brains evaluated 2 days following injury.

RESULTS

This study demonstrates that an FP-induced cortical lesion can be assessed and quantified using TTC. The uninjured cortex stained uniformly red with TTC, and the border between the red (viable) tissue and white (injured) tissue was easily detected in the injured cortex. The subcortical white matter did not stain in either injured or uninjured/control animals. A relatively small number of sections had an area of “pink” tissue (indicative of compromised mitochondrial respiratory activity) bordering the injured white tissue, which was included as part of the “lesion” when calculating total lesion area. Representative coronal brain sections are shown in Figure 1 to illustrate the region of cortical lesion following FP brain injury of low, moderate, and high severity. The calculated lesion volume was significantly correlated with the severity of brain injury (r = 0.85, p < 0.001) (Fig. 2A). Sham (uninjured) animals had no detectable cortical lesion (0 mm³). The mild, moderate, and high injury groups demonstrated lesion volumes of 12.1 ± 4.5 mm³ (mean ± SD), 33.8 ± 8.6 mm³, and 45.1 ± 14.0 mm³, respectively (Fig. 2B). Statistical evaluation using an analysis of variance (ANOVA) demonstrated a highly significant difference between these groups (p < 0.001). A t test with Bonferroni correction verified significance between all injury groups (p < 0.05). A portion of the increased lesion volume observed with higher injury severity appeared to be due to an increase in the rostral-caudal extent of the cortical injury, as illustrated in Figure 3.

Using H&E staining, we confirmed that the ipsilateral cortex and the subcortical white matter show damage at 48 h postinjury in animals at moderate and high injury severities. Macrophages were primarily localized in the core of the lesion, while glial cells were identified primarily in the perifocal injured tissue. The relative density of macrophages was greater than that of glial cells, yet was apparently not great enough to result in detectable levels of TTC staining (data not shown). The core of the lesion site was consistently white following TTC staining in injured animals. The extent and location of the lesion when measured using this TTC staining technique closely corresponded to that observed using conventional Nissl staining at 1 week following injury (Fig. 4). It is important to note that the lesion identified by toluidine blue is virtually identical to those previously characterized (Cortez et al., 1989). Lesion volume calculated using TTC staining for animals injured at a moderate severity was 33.8 ± 8.6 mm³, while lesion volume calculated using conventional Nissl staining was 30.4 ± 11.1 mm³.
FIG. 1. Representative coronal brain sections taken from animals following sham injury, mild, moderate, and severe FP brain injury in the rat. TTC staining following brain injury of low, moderate, and high severity demonstrated increasing areas of injured tissue (illustrated by asymmetrical white areas in the left cortex). Note that subcortical white matter does not stain with TTC even in sham (uninjured) animals.

DISCUSSION

In the present study we have modified and successfully characterized a staining technique for detection and quantification of lesion volume using TTC, in rats subjected to lateral FP brain injury of graded severity. To our knowledge, this is the first report regarding the utility of TTC staining to quantify lesion volume in a model of experimental brain trauma. Our results demonstrate that lesion volume directly correlates with increased injury severity.

The technique for staining injured brain tissue using TTC was developed due to its ability to yield a clear and accurate delineation between viable and damaged tissue. We found staining with TTC to be easier and more rapid than conventional histological assessments of lesion volume. Using the technique described in this study, it takes approximately 20 min to obtain the total cerebral lesion volume in one animal brain from the time of decapitation. Only a few previous studies have attempted quantitative evaluation of lesion volume following fluid-percussion brain injury. In one study, H&E staining was used to demonstrate a reduction in lesion volume following administration of the glutamate antagonist Eliprodil (Touliomond et al., 1995). A more recent study by Dietrich and colleagues has shown reduction in cortical damage using routine histopathologic analyses (Dietrich et al., 1994a). These encouraging studies underscore the need to develop a standardized technique to evaluate lesion volume following experimental brain trauma. TTC staining may permit a more accurate delineation of lesion volume when compared to H&E staining. Moreover, it has been estimated that tissue staining with H&E is more labor intensive and costly than TTC staining (Bederson et
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FIG. 2. Lesion volume versus injury severity following lateral FP brain injury in the rat. (A) Linear regression analysis demonstrated that a significant increase in lesion volume (mm³) was seen as the injury severity was increased (r = 0.85); and (B) lesion volume grouped according to injury severity [low injury (1.1–1.3 atm); moderate injury (2.0–2.3 atm); and high injury (2.4–2.6 atm)] also demonstrated a significant difference between groups (*p < 0.05). Bars represent means; vertical lines, one standard deviation.

al., 1986). However, a disadvantage of the TTC technique is that it does not reveal selective neuronal necrosis or other subtle histopathologic changes.

While protocols from previous work with models of cerebral ischemia proved helpful to us in the application of TTC staining in experimental neurotrauma, some modification of the technique was necessary to achieve optimal staining. In a series of preliminary studies, we observed that TTC administration via transcardiac perfusion resulted in inconsistent staining when compared to the tissue immersion technique (data not shown). Intraparenchymal hemorrhage and blood–brain barrier disruption following experimental TBI results in heterogeneous staining of viable tissue using the vascular perfusion technique. Moreover, the immersion technique described by Isayama et al. (1991) was modified in our laboratory by immediately placing glass slides over the tissue for the 7 min staining period and by maintaining all solutions at 37°C using a heating pad. The glass allows more homogeneous absorption of TTC into the tissue, perhaps by maintaining a uniform thickness of the section during staining. The use of 1-mm-thick sections in our study, as opposed to more conventional 2-mm sections (Isayama et al., 1991), may also be partially responsible for the faster and more thorough absorption of TTC into each section. Even though the number of sections was greater, the total incubation time was less using 1-mm-thick sections and this also permits more precise calculation of the lesion volume.

Unlike studies with models of focal cerebral ischemia, we do not feel that general physiologic responses to injury may have played an important role in our TTC findings. Although physiologic variables were not evaluated in this study, many previous studies in our laboratory have demonstrated only very minute physiologic changes following injury at the same severity as utilized in the present study. Temporalis muscle temperature, reflecting brain temperature, has been shown not to change in sham animals, while FP brain injury modestly lowers temperature by 0.3–0.6°C for less than 30 min following injury (Yamakami and McIntosh, 1989; Okiyama et al., 1992, 1994; Smith et al., 1993). In addition, these studies have demonstrated that MABP, and arterial Pao₂, Paco₂, and pH have been shown to remain relatively unchanged for at least 90 min following injury.

Using light and electron microscopy, mitochondria have been reported to remain intact for as long as 6 h following decapitation (Liszczak et al., 1984). This observation might help to clarify the lack of reproducibility between TTC staining and H&E quantification of lesion volume at timepoints less than 6 h following ischemic insults (Bederson et al., 1986; Cole et al., 1990; Isayama et al., 1991; Minematsu et al., 1992). Measurement of lesion volume by TTC staining at these early timepoints may be problematic in models of experimental TBI as well, since mitochondrial oxidative capacity was found to be unimpaired, with no significant loss of ATP, up to 4 h after lateral FP brain injury of moderate severity (Vink et al., 1990, 1994). Using 2-deoxyglucose autoradiography, an immediate increase in local cerebral metabolic rate for glucose was reported to occur after FP brain injury in the rat; the rate then decreased, reaching hypometabolic values by 6 h postinjury (Yoshino et al., 1991). These results suggest that approximately 6 h postinjury may represent a critical timepoint for onset of mitochondrial failure. Thus, the timepoint chosen for evaluation is important to consider because TTC may not accurately stain injured tissue at acute timepoints. Recent preliminary studies using optical fluorescence to map mitochondrial redox states have qualitatively demonstrated the absence of oxidative metabolism following lateral FP
injury (Smith et al., 1994) in identical regions of the brain, which remain white when stained with TTC in the present study, further suggesting that TTC staining may be useful as a marker of metabolically mediated cellular injury. Additionally, histological assessment in the present study suggested that both the relative location and extent of the lesions evaluated with TTC at 48 h following injury were comparable to the evolved necrotic lesion shown by toluidine blue staining at 1 week following injury. This suggests that the use of TTC staining techniques may be useful in delineating the area of injured tissue, which is destined to undergo cell death or degeneration.

At 36 h posts ischemia, macrophages have been observed to invade the injured tissue and, having mitochondria of their own, stain the subcortical white matter (Liszczak et al., 1984). Using H&E histology, we investigated whether or not the presence of macrophages or
glial cells may have impacted upon our TTC staining. Macrophages in the core of the lesion and a limited number of glial cells in the perifocal tissue, or "penumbra," were noted. These findings are consistent with others who have characterized the infiltration of macrophages, neutrophils, and glial cells into the injury site following lateral FP brain injury (Cortez et al., 1989; Hill et al., 1993; Soares et al., 1995). Despite the presence of macrophages in the site of maximal tissue damage, the observation that this area did not demonstrate detectable TTC staining suggests that the presence of macrophages, leukocytes, or reactive glial cells did not result in an underestimation of lesion volume.

Our observations support the potential utility of TTC staining as an endpoint to quantify lesion size in future studies of experimental brain injury. This TTC staining technique for quantifying cellular damage is rapid, easy, and accurate. This technique should prove useful in the evaluation of neuroprotective therapies in models of CNS trauma.

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Address reprint requests to: Tracy K. McIntosh, Ph.D. Division of Neurosurgery University of Pennsylvania 3320 Smith Walk/105 Hayden Hall Philadelphia, PA 19104-6316