Neurotoxicity of 1,3,5-Trinitrobenzene (TNB): Immunohistochemical Study of Cerebrovascular Permeability


Department of Anatomy, Pathology, and Pharmacology, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK (AMSC, GAC, CWQ); and US Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD (GR)

Abstract. 1,3,5-Trinitrobenzene (TNB) is a soil and water contaminant at certain military installations. Encephalopathy in rats given 10 daily oral doses of TNB has been reported. The lesion was bilaterally symmetric vacuolation and microcavitation in the cerebellar roof nuclei, vestibular nuclei, olivary nuclei, and inferior colliculi. The contribution of the blood–brain barrier (BBB) in the genesis of these lesions remains uncertain. One of the main goals of the present work was to evaluate the functional state of the BBB. Male Fischer 344 rats (five rats/group) were euthanatized after four, five, six, seven, eight, or 10 daily doses of TNB (71 mg/kg). A different set of rats (five rats/group) was allowed to recover for 10 or 30 days after receiving 10 doses of TNB. Integrity of the BBB was assessed by immunohistochemical staining for extravasated plasma albumin on paraffin-embedded sections. Rats euthanatized after four to eight doses had no lesions, and albumin extravasation in the susceptible regions of the brain was minimal. Rats receiving 10 daily doses of TNB had bilaterally symmetric vacuolation and microcavitation in the cerebellar nuclei, vestibular nuclei, and inferior colliculi in association with multifocal, often confluent foci of extravasated albumin in susceptible nuclei. Albumin was present in vascular walls, extracellular space, and neurons. Immunoreactivity in neurons was of two types: cytoplasmic staining representing pinocytic uptake and homogenous staining of the entire neuron (nucleus and cytoplasm) due to uncontrolled albumin leakage through the damaged cell membrane. In rats allowed to recover for 10 days, the microcavitated foci were infiltrated by glial and gitter cells. Albumin immunoreactivity was present as extracellular granular debris, and neuronal staining (for albumin) was mild. In rats allowed to recover for 30 days, immunoreactivity to albumin was not seen. This study demonstrates that TNB-mediated tissue damage is accompanied by breakdown of the BBB. The presence of vacuolation and associated extravasated serum proteins in TNB-treated rats is an indication of vasogenic brain edema, which appears to be a critical event in TNB toxicity. Additional studies are needed to determine the reason for selective regional vulnerability and brain microvasculature susceptibility to TNB.

Keywords: Albumin; blood–brain barrier; immunohistochemistry; munitions; neurotoxicity; rats; 1,3,5-trinitrobenzene.
tributes to the injury. This association between plasma leakage (vasogenic edema) and nerve cell death has been demonstrated in several animal studies. For example, opening of the BBB by intracarotid infusion of a hyperosmolar solution caused nerve cell injury in areas where leakage of the BBB was evident from extravasation of plasma proteins. Cytolytic neurodegeneration was found in areas that showed protein leakage in stroke-prone hypertensive rats. Short-lasting (transient) BBB opening induced by adrenalin infusion, aortic clamping, epileptic seizures, or cerebral infarction causes neuronal damage with a spatial relationship to the extravasation of plasma proteins. The results of these studies suggest that if the BBB is breached, the extravasated plasma components may be neurotoxic.

The main goals of the present work were to evaluate the functional state of the BBB and to determine if a correlation exists between BBB dysfunction and the lesions observed with TNB. A breached BBB can be detected by immunohistochemical staining of an endogenous protein, such as albumin, or of an exogenously administered substance, such as horseradish peroxidase (HRP) or Evans blue dye. Immunohistochemical staining for albumin in histologic sections is a well-established method for demonstrating extravasation and confirming vasogenic edema.

Materials and Methods

Chemicals

TNB (99.83% purity) was obtained from the US Naval Surface Warfare Center (Silver Spring, MD), and the purity of the compound was confirmed by high-performance liquid chromatography. TNB was ground to a fine powder in a Potter-Elvehjem grinder and then mixed with corn oil to form a solution. The TNB/corn oil mixture was prepared daily just prior to dosing. The oral LD50 value for TNB in rats is 284 mg/kg (for combined sexes). The 25% LD50 dose (71 mg/kg) was selected for this study because brain damage was not evident after repetitive administration of the 12.5% LD50 dose (35.5 mg/kg) in a previous experiment.

Animals

After a 2-week acclimatization to laboratory conditions (12 hours light/12 hours dark cycle, 22–24 C, 50% ± 10% relative humidity), male Fischer 344 rats (Charles River, Raleigh, NC) were weighed and assigned to seven experimental groups. The animals were housed one per cage and were provided with Purina Laboratory Chow (Ralston Purina, St. Louis, MO) and tap water ad libitum.

Experimental design

Rats were randomly assigned by body weight to seven experimental groups (five rats/group). Within each group, four rats were gavaged with 71 mg/kg of TNB, and the control rat was gavaged with corn oil (vehicle). All rats were monitored daily for clinical signs of neurotoxicity. Rats in groups I, II, III, IV, and V, were euthanatized 24 hours after final administration of four, five, six, eight, or 10 consecutive daily doses of TNB, respectively. The remaining groups received 10 consecutive daily oral doses of TNB (at 71 mg/kg) and were euthanatized after recovery periods of 10 (group VI) or 30 (group VII) days. Rats were deeply anesthetized with sodium pentobarbital (20–30 mg/kg) and perfused transcardially with neutral buffered 10% formalin. The skull was opened, and the brain was fixed in situ until the next day, when it was removed from the skull and immersed again in fresh fixative.

Histologic and immunohistochemical staining procedures

After removal, each brain was carefully sectioned (rostral to caudal) into 2.0-mm-thick coronal slices, using a rat brain matrix and a sharp razor blade, for macroscopic examination. The examined regions included telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. The brains slices were then dehydrated, routinely processed, and embedded in paraffin. Paraffin-embedded sections were cut at 4–6 μm and stained with hematoxylin and eosin (HE). To minimize variability among rats, the examined sections were standardized so that they were all from approximately the middle of the susceptible region. Serial sections of the brain were prepared in parallel for immunohistochemical studies. Step sections were cut (saving one in 10), and only consecutive sections (exhibiting the lesion from any given rat) were stained with HE and immunohistochemically for albumin.

For immunohistochemical staining, paraffin-embedded 4–6-μm-thick sections were placed onto poly-L-lysine-coated slides and dried overnight (37 C). On the following day, the sections were deparaffinized in xylene and hydrated via a graded ethanol series. All steps were conducted at ambient room temperature. Endogenous peroxidase activity was blocked with a 30-minute incubation in methanol containing 3% hydrogen peroxide. Between the remaining steps, sections were washed twice for 10 minutes each in isotonic phosphate-buffered saline (pH 7.4). Sections were preincubated for 30 minutes in normal goat serum to decrease nonspecific binding. Excess serum was blotted before primary antibody application. For the determination of extravasated serum albumin, sections were incubated with rabbit anti-rat albumin (1 : 16,000) (Cappel, Organon Teknika Corp., Durham, NC) for 60 minutes. The bound primary antibodies were visualized with a avidin–biotin–peroxidase complex kit (Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA). Immunolabeled peroxidase was visualized with the purple chromogen Vector VIP (Vector Laboratories). Harris’s hematoxylin was used as a light nuclear counterstain. After a brief rinse with tap water, sections were dehydrated and coverslipped. To validate the specificity of the antibody, primary antibody was adsorbed with excess purified antigen (rat albumin); incubation of sections with the adsorbed antibody completely eliminated immunoreactivity (in control and treated rats). In addition, specificity of the immunohistochemical reaction was evaluated by the substitution of normal rabbit serum for the primary antibody. Sections from all rats were immunohistochemically stained on the same day.
using the same diluted antibody solution throughout to avoid variation in staining between sections and to minimize variability within different experimental groups.

Results

Clinical signs

The earliest clinical signs were observed approximately 30 minutes after dosing. The TNB-treated rats showed mental depression, rapid breathing, and cyanotic ears, eyes, and feet. The cyanosis was due to the methemoglobinemia induced by TNB, as previously reported. These clinical signs subsided 2–3 hours after dosing. In addition to methemoglobinemia, clinical signs of CNS neurotoxicity (groups V, VI, and VII) ranged from mild head shaking to walking on toes and (in the more severe cases) pivotal movement (keeping the rear legs stationary while moving in semicircles with the forelegs). During the recovery phase, rats in groups VI and VII had minimal clinical signs only on days 11–13, including dullness, lethargy, and subtle tremors.

General histology

The brains of all control rats were histologically normal, as were the brains from rats euthanatized after four, five, six, and eight doses (i.e., groups I–IV) of TNB. Rats euthanatized after 10 daily doses (group V) had vacuolization and microcavitation of the neuropil and widened Virchow-Robin spaces containing erythrocytes. These previously described lesions were confined to the cerebellar roof nuclei, medial and lateral vestibular nuclei, olivary nuclei, and inferior colliculi. In rats euthanatized after a 10-day recovery period (group VI), the same regions were affected, exhibiting a focally dense infiltrate of foamy macrophages and glial cells (Figs. 1, 2). Occasional shrunken, intensely eosinophilic angular (necrotic) neurons and axonal spheroids were observed in and around the resolving foci. A few scattered Purkinje cells were intensely eosinophilic and shrunken, with a clear halo around the cell boundary. In the group VII rats euthanatized after a 30-day recovery period, vacuolation of the neuropil and infiltrates of macrophages and glial cells were minimal. A few neurons in the susceptible nuclei were necrotic (Fig. 3), and mild scattered, loss of Purkinje cells was evident in the cerebellar folia. The loss of Purkinje cells was not uniform or diffuse; normal Purkinje cells were occasionally present next to degenerate or lost cells (Fig. 4). Rarely, isolated cerebellar granular cells were also necrotic.

Immunohistochemistry

The brains of all control rats had immunoreactive albumin within (intraluminal) meningeal vessels, choroid plexus, and rarely capillaries, small venules, and arterioles in the neuropil (Fig. 5). Albumin was not present extravascularly. Rats euthanatized after four, five, and six doses of TNB (groups I, II, III) had a staining pattern identical to that of the control rats. In two of four rats euthanatized after eight doses of TNB (group IV), immunoreactive albumin was faintly detected as extravascular, extracellular punctate deposits around the olivary nuclei and within the inferior colliculi. Staining of neurons and glial cells was not observed.

All rats euthanatized after 10 daily doses of TNB (group V) had a marked increase in the intensity and extravascular distribution of immunoreactive albumin. Widespread areas of purple reaction product were seen extracellularly and intracellularly in the cerebellar peduncle, inferior colliculi, and brain stem (Fig. 6). The extravasation of albumin was not confined solely to those regions having vacuolated appearance. Instead, the reaction product had a centrifugal spread to a limited distance into the white matter of the adjacent cerebellar folia and diffusely throughout the brain stem. Similar to the control rats, albumin immunoreactivity was present in the meningeal vessels and in the choroid plexus (internal positive control). Numerous vessels, particularly small arterioles and veins, contained extensive immunoreactive albumin in the vessel wall and as halos in the perivascular spaces (Fig. 7). Immunoreactivity in the arterioles was particularly pronounced in those regions of the brain susceptible to damage by TNB (cerebellar roof nuclei, inferior colliculi, and olivary nuclei). The neurons within and adjacent to the vaculated foci contained albumin, which varied between individual cells. Some neurons con-
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Figure 1

Figure 2

Figure 3

Figure 4
tained fine purple granules exclusively in the cytoplasm, sparing the nucleus and nucleolus (cytoplasmic labeling) (Fig. 8). These neurons had normal shape and size. The other pattern was observed in the necrotic neurons, wherein immunoreactivity completely obliterated nuclear and cytoplasmic details (homogeneous labeling). Immunoreactivity to albumin was present in some glial cells (interpreted as astrocytes and microglia) in these foci; however, this finding was inconsistent among rats. In addition to the neurons in the vacuolated foci, the cerebellar Purkinje cells also had both cytoplasmic and homogeneous labeling patterns (Fig. 9). The Purkinje cell staining was confined only to those cells in the immediate vicinity of the cerebellar roof nuclei. The cerebellar granule cells and the molecular layer did not stain positively for albumin, even though the Purkinje cells and the white matter were positive. Immunoreactivity to albumin was not detected in the cerebral cortex in any rat.

In contrast to the group V rats, group VI rats (i.e., those euthanatized after a 10-day recovery period) had albumin immunoreactivity that was no longer present as a diffuse pattern in neuropil. Immunoreactivity to albumin in these rats appeared as speckled granular purple debris between and within the numerous macrophages, glial cells, and new capillaries. The staining intensity of neurons varied among the rats, with some showing intense and others faint immunoreactivity to albumin. Rats given a 30-day recovery period (group VII) had no extravascular in the susceptible regions of the brain, a pattern comparable to that of control rats.

**Discussion**

This study involved examination of the role of the vascular bed in the genesis of the symmetrical brain stem lesion produced by TNB and other nitroaromatic compounds. The TNB-induced encephalopathy is accompanied by vacuolation that is confined to the cerebellar roof nuclei, vestibular nuclei, inferior colliculi, and olivary nuclei. These histologic changes are largely responsible for the neurologic signs exhibited by affected rats because the cerebellum is an integration center of posture and voluntary movement. The vacuolation observed with TNB and other nitroaromatic compounds suggested vasogenic edema, but vacuolation has also been associated with cytotoxic brain edema. Immunohistochemical demonstration of extravasated serum proteins confirms the occur-

**Fig. 5.** Brain; control rat. Immunoreactivity to albumin is confined to the choroid plexus (fourth ventricle) and capillaries in the neuropil. There is no extravascular immunoreactivity. Compare with Figs. 6 and 8. Anti-rat albumin, ABC immunoperoxidase method, hematoxylin counterstain. Bar = 20 μm.

**Fig. 6.** Brain; rat. Antialbumin staining in the inferior colliculus after 10 doses of TNB. There is marked immunoreactivity within and around the blood vessels and diffusely in the neuropil. Note the adjacent sharply delineated, unstained cerebral cortex. Anti-rat albumin, ABC immunoperoxidase method, hematoxylin counterstain. Bar = 20 μm.

**Fig. 7.** Brain; rat. Higher magnification of Fig. 6 showing pronounced albumin immunoreactivity in vessels after 10 doses of TNB. Arrowheads indicate albumin-positive necrotic neurons. Anti-rat albumin, ABC immunoperoxidase method, hematoxylin counterstain. Bar = 50 μm.

**Fig. 8.** Brain; rat. Immunostained neurons from the olivary nucleus after 10 doses of TNB. Note the cytoplasmic (arrows) and homogeneous (arrowheads) staining of the neurons. Anti-rat albumin, ABC immunoperoxidase method, hematoxylin counterstain. Bar = 50 μm.
rence of vasogenic edema as opposed to cytotoxic edema, in which no protein extravasation is present.\(^{31}\)

### BBB and extravasation of plasma proteins

The regional distribution of the TNB-induced lesions was identified with the immunohistochemical stain for extravasated serum albumin as a method of evaluating the continuity of the BBB. The immunohistochemical method is considered the most sensitive one available for the detection of vascular permeability and is thought to reflect the accumulation of extravasated serum albumin up to the time when animals are euthanized.\(^{4,10}\) The results of this study indicate a transient opening of the BBB (increased vascular permeability) in rats treated with TNB for 10 days. Similarly, transient opening of the BBB has been reported with infusion of hyperosmolar solutions,\(^{34}\) hypertension,\(^{8,35}\) adrenalin-induced hypertension,\(^{36}\) epileptic seizures,\(^{9,23}\) cerebral ischemia,\(^{10,18}\) and bicuculline-induced experimental seizures.\(^{37}\)

After 10 days of recovery, the immunoreactivity was present as granular debris, and at 30 days post-treatment, immunoreactivity was confined to the choroid plexus and meninges (similar to the control rats). These results indicate a complete restoration of the BBB. Identical results were obtained elsewhere, where extravasated serum albumin was not detected in rats 7 days after an episode of acute hypertension,\(^{35}\) in rats subjected to experimental seizures with bicuculline,\(^{37}\) and in gerbils after cerebral ischemia.\(^{10}\) Studies with isotope-labeled albumin indicated that extravasated proteins are cleared from the brain within a short time after a transient opening of the BBB.\(^{21}\)

The mechanism of action of TNB is not known. Endothelial cell damage or a frank vascular rupture was not observed in this or a previous study.\(^{2}\) Ongoing in vitro studies with endothelial cells also have not indicated evidence of cytotoxicity (unpublished data). Very close similarities exist between TNB-induced encephalopathy in rats and pyrithiamine-induced encephalopathy (Wernicke’s encephalopathy) in mice.\(^{41}\) Hemorrhagic lesions and increased vascular permeability (to HRP) were observed in these mice.\(^{42}\) However, transmission electron microscopy revealed intact endothelial cells and tight junctions. To explain these phenomena, a transendothelial transport was suggested as the likely route.\(^{42}\)

### Fate of extravasated proteins

Neurons, including Purkinje cells, were prime targets for the extravasated proteins. The neuronal uptake of albumin was usually seen within and close to points of extravasation, but strongly positive neurons (including Purkinje cells) were also present outside the actual leakage sites. Accumulation of albumin and protein tracers in neurons has been observed repeatedly in various experimental models of BBB damage.\(^{1,3,5,7,23,26,28,29,31,34–36,39,40}\) The distribution of albumin within the neurons in the present study was similar to that in other reports.\(^{17–20,34–36}\) At the edge of the lesion, neuronal staining for albumin was of two types. The albumin immunostaining in some neurons was homogeneous, obscuring cytoplasmic and nuclear details, whereas in other neurons the staining was only cytoplasmic. Neurons with cytoplasmic albumin may represent living cells that have accumulated proteins in lysosomes after vesicular transfer across the plasma membrane.\(^{13,14,16,19,22,39}\) whereas the diffuse form is likely the result of a protein influx in a cell with a severe cell membrane injury.\(^{5,12,28,40}\) In these studies, it was suggested that transient opening of the BBB may have induced neuronal damage. Mechanisms proposed to explain neuronal staining include 1) passive diffusion through the damaged membrane as observed in the dead neurons, 2) damaged neuronal processes, and 3) active uptake to reduce the vasogenic edema.\(^{10}\) The cells that show cytoplasmic immunostaining with intact nuclei appeared to be viable and responding actively to the extravasation. If the BBB opening persists for a longer period, these neurons could become irreversibly damaged.\(^{10}\)

TNB-mediated tissue damage is accompanied by concurrent breakdown of the BBB, which suggests that regional vasculature plays an important role in the genesis of the lesion. Further studies are needed to uncover other factors determining the selective vulnerability of some regions and the mechanism by which TNB promotes increased vascular permeability.

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