1,3,5-TRINITROBENZENE – INDUCED ENCEPHALOPATHY IN MALE FISCHER-344 RATS

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2000

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ENCEPHALOPATHY IN MALE

FISCHER-344 RATS

Thesis Approved:

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PREFACE

This dissertation is a study of the neurotoxic effects of 1,3,5-trinitrobenzene in male Fischer-344 rats. This compound is commonly produced during the manufacture of munitions, particularly 2,4,6-trinitrotoluene. Concern over release of this chemical into the environment has resulted in studies to illuminate possible mammalian risks from exposure. Current information regarding this compound has relied heavily on known toxicity data from structurally similar nitroaromatic compounds and previous work by my colleagues. While working on this project, I relied heavily upon multiple published reports and communications from other researchers who are or have been involved with this compound. The purpose of this study was to concentrate on the neurotoxic effects that had previously been described by Dr. Sundeep Chandra in his Ph.D. dissertation. I wanted to reproduce the neurotoxicosis described by Dr. Chandra, ensure that it was neurotoxic and try to elucidate some possible mechanisms. The first phase of these studies (Chapter II) concentrated on the possible role of nitric oxide and thiamine in producing or decreasing the neurotoxicosis *in vivo*. The next phase of my studies (Chapter III) concentrated on the effects of parentally administered anti-oxidants (vitamin E, vitamin C, and N-acetylcysteine) in an attempt to prevent or decrease the neurotoxic lesions induced by 1,3,5-trinitrobenzene exposure. The last phase of these studies (Chapter IV & V) concentrated on discovery of a toxic dose to astrocytes and endothelial cells comprising the blood brain barrier.

This work is the result of my labors with the help of many friends, family, and the support of my department. I am most indebted to Dr. Charles W. Qualls Jr., my initial major professor, for his guidance and encouragement during my thesis research. I chose Chuck

as my advisor because of his vast knowledge and interest in toxicologic mechanisms as well as his strong background in pathology. During my training, I have found him to not only be an outstanding scientist but to also have a keen sense of humor. He has been a great help in not only finding funding for my research, but also is assisting me in searching for employment beyond my graduate experience. I would also like to express my sincere gratitude to Dr. Subbiah Sangiah, my final major professor who graciously took me on as his student when Dr. Qualls left Oklahoma State University. Dr. Sangiah was instrumental in working with me though many difficulties associated with the completion of this dissertation. He was very patient during the difficult task of putting together the many pieces of this "big picture". Dr. Sangiah, I also want you to know that I not only considered you one of my mentors, but a friend who I could count on when I was feeling overwhelmed and discouraged. I would also like to thank the other members of my graduate committee, Dr. Gunda Reddy, Dr. W.C. Edwards and Dr. Greg Campbell for their help, support and encouragement.

Much of the financial support for this study was provided by a research grant from the Health Effects Research Division of the U.S. Army Center for Health Promotion and Preventative Medicine. Special thanks go out to Dr. Gunda Reddy from this organization, for his help and advice through all stages of this project. I would like to thank the Environmental Institute at Oklahoma State University for providing me with the presidential fellowship for water, energy, and the environment to help support my research. I would also like to thank the Department of Anatomy, Pathology and Pharmacology as well as the staff of the Oklahoma Animal Disease Diagnostic Laboratory for all of their assistance. Special thanks to fellow graduate students, Dr. Soochong Kim, Dr. Nick Gatto, Dr. Karamjeet Pander, Dr. Sundeep Chandra, and Dr. Sabu Kuruvilla for all their assistance with my experimentation and the many laughs they provided during and after hours. I owe Dr. Jerry Ritchey and Dr. Jeremiah Saliki many thanks for the use of their laboratories during my *in vitro* studies. Dr.

Saliki and his staff at the diagnostic lab were instrumental in my gaining experience with handling cells in culture and to them I am much indebted.

I owe an enormous amount of thanks to one of my best friends, Dr. Joe Roder, who has always been there when I needed him. His encouragement and assistance were instrumental in the completion of this specialty training. Without Joe's assistance, I'm sure I would not have completed this training. He was never too busy to listen to me bitch or walk me through an experiment. We studied and passed the ABVT examination together in Louisville, just as he earlier professed. So, Joe, I will always have a candle lit for you, you have been like a brother to me, thank you very much.

The greatest acknowledgment I would like to give for assistance with the completion of this journey goes to my family. I was raised in a home with a loving father and mother who both had their own significant academic accomplishments. The pride I had for both my parent's achievements provided some of the impetus for my undertaking this goal. I was fortunate to be able to live at "home" during the majority of my training. Living with my folks removed any possible financial strains and provided me with significant mental stability. My mother, Eileen, was always available to lend a hand or an ear with problems throughout my education. Thanks mom, for all of your support. My father, Dr. Ernest Stair Jr., was instrumental in my entering the field of veterinary medicine. I often accompanied him during his Ph.D. research to prevent me from fighting with my brother Evan. I feel these trips to work with "dad" laid the groundwork for my interest in science and eventually veterinary medicine. Dad, thanks for all your help and patience through the years, and for taking the time from your busy schedule to always help me when I needed it! My brothers Eron and Evan and my sister-in-law Dana also deserve special credit. Evan always listened to my problems and has kept me laughing to take my mind off my troubles. Evan's wife Dana assisted me by photocopying journal articles for me from the University of Oklahoma library. Eron helped me in many ways throughout this project for which I owe him many thanks.

Whether assisting with animal treatments or typing pathology reports from recorded dictation, he always helped me without complaint.

I'm sure I've probably forgotten to include thanking someone for their help, so this paragraph goes out to anyone I may have left out, THANKS!

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CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction

Humans and other mammals are frequently at risk from exposure to hazardous environmental and industrial chemicals. These chemicals have the potential to inflict many toxicologic and pathologic effects. Harmful effects are dependent on many factors including route of exposure, amount or dose of chemical the subject is exposed to, and duration or frequency of exposure. Other factors such as individual differences in response, potential interactions between chemicals, and whether or not the toxic effects are reversible all play a role in determining toxicologic and pathologic sequela.

Toxicologic responses of the nervous systems of mammals have been observed and recorded from exposure to environmental and industrial chemicals. In fact, many advances in the understanding of neurobiology have come from observations of toxic insults on nervous tissue. Generalities about the mammalian nervous system must be understood to gain a grasp of how neurotoxicants may inflict damage. These general principles include: (1) the privileged status of the nervous system that maintains the blood-brain barrier, (2) the specific energy requirements of the brain, (3) the complex shapes of nerve cells with relation to their spatial extensions in the nervous system, (4) maintaining an environment rich in lipids, and (5) the transmission of information across an extracellular space (Anthony et al., 1996).

Nitroaromatic compounds are extremely important to the chemical industry. Nations will continue to manufacture and dispose of large quantities of these chemicals that have been used to make many consumer and industrial products. Human and animal exposure to a variety of these compounds has resulted in toxicologic consequences including neurotoxicosis. A review of the literature regarding nitroaromatic compounds with regards to their history, chemistry, and biological effects is warranted. This dissertation will focus on examining the neurotoxic effects associated with a specific nitroaromatic compound, 1,3,5-trinitrobenzene.

History of Nitroaromatic Compounds

The chemical industry is very dependent on the production of nitroaromatic compounds. The earliest use of nitroaromatics dates back to the late 19^{th} and early 20^{th} century with the discovery and utilization of several distinct explosives. Later, synthesis of industrial chemicals included pesticides, polymers of plastic and rubber, and some pharmaceutical products.

Nitroaromatic compounds are the chemical industries only source of aromatics with nitrogen bound to the benzene ring (Hartter, 1985). Large markets have developed for the manufacture and use of these chemicals. The versatility and utilization of nitroaromatics is substantial and should continue well into the future.

Chemistry of Nitroaromatic Compounds

All nitroaromatic compounds have the basic chemical structure of a nitro ($-NO_2^-$) group attached to an aromatic ring. The aromatic ring is composed of unsaturated carbon atoms, such as benzene, naphthalene, anthracene, and their derivatives. Figures 1-4 show the molecular structures for selected important nitroaromatic compounds (nitrobenzene, trinitrotoluene, 1,3-dinitrobenzene and 1,3,5-trinitrobenzene).

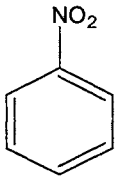

Figure 1. Nitrobenzene

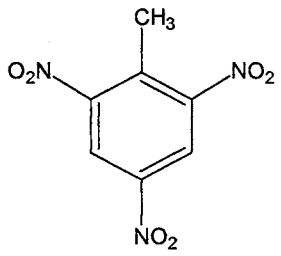

Figure 2. 2,4,6-Trinitrotoluene

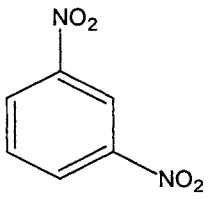

Figure 3. 1,3-Dinitrobenzene

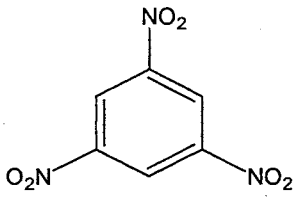

Figure 4. 1,3,5-Trinitrobenzene

These four nitroaromatic compounds are important and have been emphasized due to their similarities in toxicity observed following exposures.

Nitrobenzene is an intermediate in the synthesis of aniline and other nitroaromatic compounds. Heating benzene with concentrated nitric acid (HNO_3) and sulfuric acid (H_2SO_4) increases the rate of the nitration reaction forming nitrobenzene. Nitrobenzene is a colorless to pale yellow oily liquid with a melting point of 6°C and a molecular weight of 123.11 (Windholz et al., 1976). Nitrobenzene is produced in large quantities and may be released to the environment in emissions and wastewater during its production and use. Nitrobenzene is also produced by the photochemical reaction of benzene with oxides of nitrogen.

2,4,6-Trinitrotoluene (TNT) is a crystalline solid with a molecular weight of 227.13 and has a melting point of 80.1°C (Windholz et al., 1976). TNT is produced by the successive nitration of toluene with nitric acid in a continuous process (Hathaway, 1985). TNT is an explosive used by the military, petroleum and demolition industries, and is also an intermediate in dyestuffs and photographic chemicals (Windholz et al., 1976).

1,3-Dinitrobenzene (DNB) has a molecular weight of 168.11 and a melting point of 89-90°C (Windholz et al., 1976). This yellowish, crystalline substance is used in the production of aniline, dyes, industrial solvents, plastics, and pesticides. DNB also has explosive properties and is an intermediate compound in the industrial production of many chemicals.

1,3,5-Trinitrobenzene (TNB) is a dimorphic solid with a molecular weight of 213.11 and forms orthorhombic bipyramidal crystals that have melting points of 121-123°C (Fedoroff et al., 1962; Budavari et al., 1989). TNB is a nitroaromatic compound produced by the oxidation of 2,4,6-trinitrotoluene or the decarboxylation of trinitrobenzoic acid (Borges, 1991). TNB is an explosive that is more powerful but less sensitive to impact than TNT (Fedoroff et al., 1962; Budavari et al., 1989). TNB is primarily know as an explosive but also has had limited use in the vulcanization of rubber (Barnhart, 1981) and as a pH indicator (Durst & Bates, 1981). TNB is a soil and water contaminant at installations that are currently or have been involved in the manufacture of explosives. Environmental degradation of TNB is difficult but may be accomplished by both sunlight and microorganisms (EPA, 1989).

Biological Effects of Nitroaromatic Compounds

The potential for mutagenic or carcinogenic responses in organisms exposed to nitroaromatic compounds, especially responses observed in the use of naphthalene's, nitrated biphenyl's, and fluorene's, has resulted in heightened interest in the risks and hazards of exposure to humans and the environment (Spanggord et al., 1985; McGregor et al., 1980). Specific mammalian organ systems affected by nitroaromatic compounds include the hematologic, reproductive and central nervous systems. The four compounds listed previously (Nitrobenzene, TNT, DNB, and TNB) all have the ability on contact to penetrate human skin. Ingestion of contaminated water or foodstuffs along with inhalation risks from toxic vapors when these compounds become volatilized are other routes of potential exposure.

Hematologic Effects

Methemoglobinemia

Nitrobenzene, TNT, DNB, and many other nitroaromatic exposures in mammals have resulted in methemoglobinemia. Methemoglobin is produced in the erythrocyte when the heme irons in hemoglobin undergo valence change from +2 (ferrous) to +3 (ferric). The resulting pigment is greenish-brown to black in color and causes the blood to appear chocolate brown instead of bright red. Methemoglobin decreases the ability of erythrocytes to carry oxygen resulting in tissue hypoxia.

Nitrobenzene-induced methemoglobinemia has been observed in many species including dogs, cats, rabbits, rats and mice, although mice appear to be less sensitive than other species (Medinsky & Irons, 1985). Methemoglobinemia observed during oral

nitrobenzene exposure requires cecal microbial metabolism of nitrobenzene (reduction to aniline through the intermediates nitrosobenzene and phenylhydroxylamine) (Reddy et al., 1976; Levin & Dent, 1982). These intermediates are known to directly produce methemoglobinemia. *In vitro*, nitrobenzene increases methemoglobin formation when incubated with native hemoglobin but not when incubated with erythrocytes (Goldstein & Rickert, 1985).

The three isomers of dinitrobenzene (1,2-DNB, 1,3-DNB and 1,4-DNB) have also been reported to induce methemoglobinemia (Facchini & Griffiths, 1981; Cossum & Rickert, 1987). While all the isomers of DNB produce variable degrees of methemoglobinemia depending on the species of animal involved, 1,4-DNB was always the most effective (Cossum & Rickert, 1987). 1,2-DNB (*o*-DNB) was the least effective isomer of dinitrobenzene causing methemoglobinemia (Watanabe et al., 1976). Also significant is the difference in degree of methemoglobinemia produced depending on the animal species. Studies performed with golden Syrian hamsters and Sprague-Dawley rats showed that substantially less methemoglobinemia was observed in the hamster than the rat. Dosage levels of 25 mg/kg 1,3-DNB resulted in peak methemoglobin levels of 15% in the hamster compared with 80% in the rat (Obasaju et al., 1991). *In vitro* studies with 1,3-DNB and its metabolites (nitroaniline, nitroacetanilide, aminoacetanilide, and diacetamidobenzene) have shown that 1,3-DNB and not its metabolites are responsible for the methemoglobinemia. The *in vitro* studies also corroborated *in vivo* work that rat red blood cell suspensions were twice as sensitive to methemoglobin formation as hamster erythrocytes (Obasaju et al., 1991).

Oral dosing of rats with 300 mg/kg/day TNT for 13 weeks has resulted in significant methemoglobinemia (Levine et al., 1984). Dogs dosed orally by capsule with

TNT at 32 mg/kg/day for 6 months also had notable methemoglobin formation (Levine et al., 1990). Mice dosed with TNT via intraperitoneal injection have also resulted in increased levels of methemoglobinemia (Shugalei et al., 1993). Humans with glucose-6-phosphate dehydrogenase deficiency that were occupationally exposed to TNT are reported to have had 1.5%-8.6% methemoglobin of total hemoglobin (Djerassi, 1998).

TNB Induced Methemoglobinemia

Exposure to TNB in male F-344 rats gavaged with dosages of 35.5 and 71 mg/kg resulted in a dose-dependent methemoglobinemia present only in blood collected 5 hours after a single dose. Minimal methemoglobinemia was also observed with daily oral dosing regimens (4 or 10 doses), but were not statistically significant from control levels 24 hours after the last dose (Chandra et al., 1995). Female and male rats fed a diet containing varying concentrations of TNB (50, 200, 400, 800, and 1200 mg/kg) for 14 days had significant methemoglobin concentrations when compared with controls from both sexes (Reddy et al., 1996). Male and female rats fed a diet containing different concentrations of TNB (400 and 800 mg/kg) for 90 days also had significant methemoglobin concentrations when compared with control animals of both sexes (Reddy et al., 1998). *In vitro* spectral changes of hemoglobin recorded during incubation with TNB confirmed methemoglobin formation and progressive denaturation of hemoglobin-forming hemichromes (Chandra et al., 1995). Hemichromes are low spin derivatives of ferrihemoglobin within the hemoglobin denaturation pathway. The *in vitro* data also supports the contention that TNB does not require intestinal microbial conversion to induce methemoglobinemia.

Hemolytic Anemia

Tissue hypoxia from nitroaromatic poisoning occurs not only from methemoglobinemia but also from hemolytic anemia. Premature destruction of erythrocytes results from denaturation of hemoglobin with resultant hemichrome formation. Hemichromes are formed following severe methemoglobinemia due to the constant presence of the nitroaromatic (oxidant). Hemichrome formation and cross-linking with band 3, an erythrocytic membrane spanning protein, result in cluster formation. Erythrocytes are then opsonized by IgG allowing recognition by macrophage Fc receptor mechanisms. Erythrophagocytosis of these opsonized red blood cells allows for rapid removal from circulation via the spleen and liver.

Anemia from nitrobenzene toxicosis has been recognized for a long time. During and after acute and chronic nitrobenzene exposure, multiple blood dyscrasias have been reported. Decreased circulating hemoglobin, erythrocyte and platelet counts along with hemolytic anemia and bone marrow hyperplasia have all been documented (Shimkin, 1939; Parkes & Neill, 1953; Schimelman et al., 1978; Shimo et al., 1994). Chronic inhalation of nitrobenzene has also been reported to induce anemia in both mice and rats (Cattley et al., 1994).

Similar to nitrobenzene, the three isomers of DNB have all been documented to induce anemia on lengthened exposure (Watanabe et al., 1976). 1,3-Dinitrobenzene in drinking water for 8 weeks (at 50, 100, 200 mg/L) or 16 weeks (at 3,8,20 mg/L) has created decreases in rat hematocrit and hemoglobin values (Cody et al., 1981). Hematologic changes in dogs administered daily or intermittent subcutaneous injections of 0.1-6.0 mg/kg of 1,3-DNB for 144 days or acute doses of 10 and 20 mg/kg have been reported as early as 1949 (Kiese, 1949a; Kiese, 1949b).

The hematologic effects of TNT have been extensively documented in humans (Hathaway, 1977; Hathaway, 1985). TNT administered in the diet of dogs (20 mg/kg), rats (0.25%) and mice (0.125%) for 13 weeks resulted in anemia, with reduced erythrocytes, hemoglobin, and hematocrit (Dilley et al., 1982). Lack of bone marrow toxicity with significant anemia when Fischer-344 rats were dosed at levels of 125 mg/kg/day for 13 weeks suggested that TNT anemia was the result of hemolysis (Levine et al., 1984). Hemolytic anemia was one of the major toxic effects observed when Beagle dogs were administered TNT by capsule at dosages of 0.5, 2, 8, or 32 mg/kg/day for 26 weeks (Levine et al., 1990).

TNB Induced Hemolytic Anemia

Significant hemolytic anemia has been reported in Fischer-344 rats gavaged with TNB at 35.5 and 71 mg/kg after 4 and 10 daily doses (Chandra et al., 1995). The observed anemia was characterized by a pronounced decrease in erythrocyte numbers, hemoglobin, and hematocrit. *In vitro* spectral changes of hemoglobin recorded during incubation with TNB confirmed methemoglobin formation and progressive denaturation of hemoglobin-forming hemichromes (Chandra et al., 1995). Hemichrome formation results in cluster formation and opsonization by IgG allowing recognition by macrophage Fc receptor mechanisms. Erythrophagocytosis of these opsonized red blood cells allows for rapid removal from circulation via the spleen and liver. A 14-day study with dosages of 50, 200, 400, 800, and 1200 mg/kg TNB and a 90-day study (66.7, 400, 800 mg/kg TNB) in the diets of both male and female Fischer-344 rats validated the hemolytic anemia induced in both sexes (Reddy et al., 1996; Reddy et al., 1998).

Renal Effects

TNB Induced Renal Effects

Male and female Fischer-344 rats dosed with 35.5 and 71 mg TNB/kg/day for 10 days and male Fischer-344 rats administered TNB at 35.5 mg/kg TNB for 20 and 30 days displayed significant increases in kidney weights. Male rats exhibited dose-related accumulation of hyaline droplets containing alpha-2u-globulin in proximal tubules. Significant increases in cell proliferation in proximal tubules were observed in male rats. Renal changes observed in TNB-treated rats appeared identical to those from other chemicals that induce alpha-2u-globulin nephropathy in male rats (Kim et al., 1997).

Reproductive Effects

The major reproductive toxicity associated with nitroaromatic compounds is testicular damage. Species differences in testicular sensitivity have been elaborated with certain nitroaromatic compounds. Female reproductive organ toxicity with nitroaromatics is inconsequential. Susceptibility to testicular toxicity is also dependent on the age of the animal.

A single oral dose of nitrobenzene (300 mg/kg) has elicited degenerative changes in the germinal epithelium of Fischer-344 rats (Bond et al., 1981). These changes were observed between one and four days after exposure. Changes consisted of necrosis of the primary and secondary spermatocytes along with the appearance of multinucleated giant cells. Necrosis and decreased numbers of spermatozoa in the epididymis were seen as early as three days after exposure (Bond et al., 1981). Similar testicular toxicity was also observed upon inhalation exposure in both rats and mice (Medinsky & Irons, 1985). Testicular changes are reversible in rats receiving single oral doses of

nitrobenzene (Bond et al., 1981). Substantial recovery (> 90% regeneration of seminiferous epithelium) was observed by 100 days after a single oral dose of nitrobenzene (Levin et al., 1988). A fivefold increase in fertility index has been reported in CD rats after nine weeks of recovery from inhalation exposure to nitrobenzene (Dodd et al., 1987).

Trinitrotoluene administered to Fischer-344 rats at 125 and 300 mg/kg/day for 13 weeks resulted in testicular atrophy characterized by degeneration of seminiferous tubular epithelium (Levine et al., 1984). Testicular atrophy was also reported in rats fed TNT in the diet for 13 weeks. The atrophy was not reversible even after a 4-week recovery period (Dilley et al., 1982).

Decreased testicular and epididymal weights have been consistently observed in rats receiving a single oral dose of 1,3-dinitrobenzene (32-50 mg/kg) (Blackburn et al., 1988; Linder et al., 1988; Rehnberg et al., 1988). Infertility due to interruption of spermatogenesis was documented at a dosage level of 3 mg/kg/day of 1,3-dinitrobenzene (5 days/week) and decreased sperm production at 1.5 mg/kg/day in Sprague-Dawley rats (Linder et al., 1986). Histologic and ultrastructural investigations indicated that the somatic Sertoli cell was the initial target site within the testicle for 1,3-DNB toxicity (Blackburn et al., 1988; Foster, 1989). The testicular toxicity of dinitrobenzene is isomer specific. A single oral dose (50 mg/kg) of 1,3-dinitrobenzene (*m*-DNB) resulted in decreased testicular weight and histopathologic changes in rats while treatment with a similar dose of the 1,2- (*o*-DNB) or 1,4- (*m*-DNB) isomers had no testicular effects (Blackburn et al., 1988). Partially reversible testicular effects were observed in rats administered 6 mg/kg of 1,3-DNB (5 d/wk for 12 weeks) followed by five-month recovery (Linder et al., 1986). Other rat studies highlighting acute effects

and long term recovery (Linder et al., 1986; Hess et al., 1988) displayed diminished epididymal sperm quality by 16 days and loss of fertilizing ability five weeks after a single oral dose (48 mg/kg) of 1,3-DNB. Normal fertilizing ability in rats was observed in most animals by five months post-treatment. Several animals did not regain normal fertilizing ability, suggesting permanent reproductive damage (Linder et al., 1990).

Species differences in sensitivity to 1,3-dinitrobenzene testicular toxicity have been documented in Sprague-Dawley rats and Golden Syrian Hamsters. Sprague-Dawley rats are much more sensitive to testicular toxicity from 1,3-dinitrobenzene than Golden Syrian hamsters. Hamsters required an intraperitoneal (IP) dose of 50 mg/kg to reach equivalent blood levels of rats receiving 25 mg/kg of 1,3-dinitrobenzene. Rats had faster initial elimination rates and higher blood levels of nitroaniline while hamsters excreted more conjugated and phenolic urinary metabolites than rats dosed with 1,3-dinitrobenzene (McEuen & Miller, 1991).

Sprague-Dawley rats display differences in susceptibility to testicular toxicity depending on animal age. Testicular damage induced by 1,3-DNB (25 mg/kg, IP) was hardly detectable in Sprague-Dawley rats, which were 31 days of age. Older rats (75 and 120 days of age) displayed moderate lesions particularly in later stages of spermatogenesis (Brown et al., 1994).

TNB Induced Testicular Effects

Testicular degeneration induced by TNB toxicosis is similar to that observed with nitrobenzene and 1,3-dinitrobenzene. However, a longer duration of administration and a greater dosage were required to produce the same lesions with TNB that were observed with DNB. TNT, another nitroaromatic testicular toxicant, requires much

greater dosage levels and duration of treatment to observe similar testicular degeneration.

Reduced testicular and epididymal weights with testicular degeneration and sperm depletion were observed in Sprague-Dawley rats receiving 800 or 400 mg of TNB for 14 days (Kinkead et al., 1995). Fischer-344 rats receiving TNB at 71 mg/kg for 4 days had necrosis and degeneration of pachytene spermatocytes with significant reduction in testicular weight. Rats dosed at 35.5 mg/kg for 10 days had severe testicular lesions consisting of degenerate and elongated spermatids, and formation of multinucleate syncytial cells in seminiferous tubules (Chandra et al., 1997). A 14-day study with dosages of 50, 200, 400, 800, and 1200 mg/kg TNB and a 90-day study (66.7, 400, 800 mg/kg TNB) in the diets of male Fischer-344 rats displayed testicular weight reduction and degeneration (Reddy et al., 1996; Reddy et al., 1998).

Studies examining the regenerative capacity of the testicle after cessation of TNB dosing have shown that the testicular damage is apparently reversible. Spermatogonia and pachytene spermatocytes labeled with proliferating cell nuclear antigen (PCNA) have been found to proliferate even in completely atrophic seminiferous tubules (Chandra et al., 1997). Damage to testicular Sertoli cells by TNB results in failure of germ cell maturation. These findings suggest that the Sertoli cell is the target in TNB induced testicular degeneration.

Neurologic Effects

The neurotoxic effects of nitroaromatic compounds have been observed in the brain. Cellular damage has not been observed histologically in the peripheral nervous system. Encephalopathy induced by nitroaromatics is characterized by widespread

vacuolation of white matter in the neuropil. Anatomic areas consistently damaged include the cerebellar peduncles and the brain stem. Neuropathology consisting of petechial hemorrhages in the brain stem, bilateral malacia and reactive gliosis in the cerebellar peduncles has been reported in rats 48 hours after treatment with a single oral dose (550 mg/kg) of nitrobenzene (Morgan et al., 1985). Likewise bilateral malacia and reactive gliosis was found in the brain of a rat that received a single dose of nitrobenzene (450 mg/kg) and was sacrificed five days later (Bond et al., 1981).

Trinitrotoluene has been reported to cause brain lesions in Fischer-344 rats receiving 300 mg/kg/day for 13 weeks. These lesions consisted of foci of vacuolation and malacia (Levine et al., 1984).

1,3-Dinitrobenzene is a nitroaromatic compound that has been extensively investigated for its induced neurotoxic effects. Similarities in brain stem lesions caused by 1,3-DNB, thiamine deficiency, and nitroimidazole toxicosis has been documented (Romero et al., 1995). Germ-free Fischer-344 rats administered a single oral dose (20 mg/kg) of 1,3-dinitrobenzene had brain lesions in the cerebellar roof, vestibular and superior olivary nuclei and the inferior colliculi of the brain (Philbert et al., 1987). Brain lesions were characterized by frequent petechial hemorrhages, with erythrocytes usually being limited to enlarged spaces of Virchow-Robin. Astrocytes, oligodendrocytes and vascular elements appeared to be the primary cellular targets of 1,3-DNB (Philbert et al., 1987). Conventional rats required repeated oral dosing of 20 mg/kg 1,3-DNB to obtain equivalent toxic effects to those seen in germ-free rats. A later study by Philbert et al (1991) using a 3 mg/kg/day dose schedule of 1,3-DNB for 2 days concentrated on vascular function and structural changes during symmetrical brain stem damage. Significant changes observed included: 1. Increased blood flow to the inferior colliculi.

2. Petechial hemorrhages in the inferior colliculi, cerebellar roof, vestibular and superior olivary nuclei. 3. Focal leakage of horseradish peroxidase (an exogenous tracer) in the brain nuclei with petechial hemorrhages. 4. Periarteriolar edema and protein leakage in these brain nuclei along with astrocytic swelling. These vascular changes suggest that they may play an important role in the pathogenesis of the brain lesions (Romero et al., 1991).

Astrocytes exposed to 1,3-DNB (0.5 mM) *in vitro* had significant decreases in reduced glutathione concentrations after a two-hour incubation. This suggested a disturbance in redox balance (Romero et al., 1995). An increase in the rate of reduction of nitroblue tetrazolium in astrocytic cell culture also suggests a free-radical mediated toxicity (Romero et al., 1995). Endothelial cells had an increased sensitivity to 1,3-DNB (0.1 mM) cytotoxicity when they were co-cultured with astrocytes in comparison to endothelial cells cultured alone. When astrocytes and endothelial cells were cultured alone, astrocytes appeared to be more sensitive to equivalent concentrations of 1,3-DNB (Romero et al., 1996). Desferrioxamine (20 mM) completely protected endothelial cells from damage by 1 mM 1,3-DNB, suggesting that hydroxyl radicals mediate a portion of DNB toxicity (Romero et al., 1996).

Several experiments have illuminated the possibility that altered neuronal activity may influence the extent and severity of the glio-vascular lesion induced by 1,3-dinitrobenzene (Ray et al., 1992; Holton et al., 1997). Rupturing the left tympanic membrane in Fischer-344 rats decreased metabolic activity of the ipsilateral cochlear nucleus while increasing activity of the contralateral inferior colliculus. Dinitrobenzene administration (10 mg/kg in 4 doses over 48 hours) resulted in a substantial reduction in morphologic changes in the ipsilateral cochlear nucleus and contralateral inferior

colliculus (Ray et al., 1992). Rats administered 1,3-DNB along with the tremorogenic pyrethroid, Bifenthrin, experienced more pronounced clinical signs along with brain stem lesions that were more severe than those observed in rats receiving 1,3-DNB alone. By contrast, rats receiving general anesthesia by either isoflurane or urethane decreased metabolic activity with subsequent decreases in severity of brain stem lesions (Holton et al., 1997).

1,3,5-Trinitrobenzene - Induced Neurotoxicosis

Studies by Chandra et al. (1995) were the first to document 1,3,5-trinitrobenzene-induced encephalopathy. The neurologic effects of TNB are quite similar to those observed with other nitroaromatic compounds. TNB induced brain lesions appear in similar locations (cerebellar peduncles, inferior colliculi and brain stem) as nitrobenzene and 1,3-DNB. However, TNB requires a greater duration of exposure than these other nitroaromatics to produce similar lesions. Fischer-344 rats gavaged with 35.5 mg/kg of TNB for 10 days did not have neurologic signs or microscopic brain lesions. Rats dosed at 71 mg/kg for one or four consecutive days did not have brain lesions at necropsy. However, all rats dosed at 71 mg/kg for 10 days had microscopic brain lesions. These rats also developed clinical neurologic signs after dosing for five to seven consecutive days. A 14-day study with dosages of 50, 200, 400, 800, and 1200 mg/kg TNB in the diets of male and female Fischer-344 rats displayed brain lesions (hemorrhage, malacia, and gliosis) in the high dose groups (Reddy et al., 1996). Lesions of TNB encephalopathy were also found to be similar to those of thiamine deficiency or pyrithiamine induced encephalopathy (Wernicke's encephalopathy) in mice (Chandra, 1995).

TNB induced brain lesions consist of malacia and petechial hemorrhage in the cerebellar peduncles, inferior colliculi and brain stem. Lesions are bilaterally symmetrical and well demarcated from the normal neuropil. Extensive vacuolation (malacia) of the white matter with dense aggregates of large macrophages (gitter cells) and gliosis characterize the lesions. Neuronal necrosis is apparent within the vacuolated lesions along with glial (microglial) cell proliferation. Hemorrhage often accompanies the malacic lesions surrounding small vessels with pronounced fibrinoid change in vessel walls (Chandra et al., 1995).

Results of an immunohistochemical reaction for extravasated serum albumin indicated that a transient opening of the blood brain barrier (BBB) occurred in rats treated with TNB (71 mg/kg) for 10 days. Extravasation of serum albumin occurred both in gray and white matter of susceptible brain regions/nuclei (Chandra et al., 1995). Immunoreactivity was present as granular debris after 10 days of recovery, and 30 days recovery; immunoreactivity was similar to control rats. These findings indicate a complete restoration of the BBB.

1,3,5-Trinitrobenzene Toxicosis

Overview

1,3,5-trinitrobenzene is a man-made nitroaromatic compound commonly produced during the manufacture of munitions and armaments, particularly 2,4,6-trinitrotoluene. Environmental contamination with TNB occurs primarily in wastewater effluent or disposal of solid TNT wastes (Ryon et al., 1984; Spanggord et al., 1985; Spalding & Fulton, 1988). Contamination of groundwater has also occurred when TNB was

generated during the synthesis of p-nitrobenzoic acid, an intermediate used for the synthesis of pharmaceuticals (Wennersten, 1980). Concern over release of TNB into the environment has resulted in studies to illuminate possible mammalian risks due to exposure. Current information regarding this compound has relied heavily on known toxicity data from structurally similar nitroaromatic compounds and previous work by my colleagues.

Clinical Signs

1,3,5-Trinitrobenzene toxicosis induces different clinical signs depending on route and duration of exposure. Absorption of TNB during its manufacture primarily occurs from direct skin contact or inhalation of dust particles. However, the primary route of environmental exposure occurs as a result of drinking contaminated water.

Oral gavage of a toxic TNB dose by Fischer-344 rats results in extreme excitement immediately following dosing. This excitement lasts approximately 10 - 15 minutes. The excitement phase in Fischer-344 rats is characterized by burrowing of heads under cage bedding and moving from one end of the cage to the other. Also, rapid rubbing of the muzzle with the front feet was consistently observed.

Severe methemoglobinemia coincides with profound depression of three-to-four hours duration following the excitement phase. Tachypnea along with cyanosis of non-haired areas of skin is apparent in rats. Orange urine that stains cage bedding is produced and eliminated. Repeated daily oral gavage of toxic doses results in continuation of these acute effects. Other clinical signs develop several days into dosing.

Subacute clinical signs are primarily neurologic. Progressive ataxia is observed with continuation of daily dosing until animals become laterally recumbent and unable to eat or drink. These neurologically impaired rats lose the ability to groom themselves with their body hair forming clumps instead of being a continuous smooth coat. Red colored crusts develop in the medial and lateral canthi of the eyes. Protruding eyes and tenting of skin confirms severe dehydration.

Clinical Pathologic Changes

The clinicopathologic changes associated with TNB induced toxicosis are primarily hematologic. Rats receiving 35.5 and 71 mg/kg TNB had 20.6 and 35.6 methemoglobin (as % total hemoglobin) five hours after a single dose. Methemoglobin levels return to normal 24 hours after a single dose. A significant dose-dependent anemia has been documented in rats that have received 4 daily doses of TNB at 35.5 mg/kg/day. The TNB induced anemia is characterized by decreased erythrocytes, hemoglobin, and hematocrit. Changes in erythrocytic size and hemoglobin concentration were also altered significantly. After 10 daily doses of TNB at 35.5 and 71 mg/kg a dose-dependent thrombocytosis, elevated nucleated erythrocyte count and significant increase in the number of reticulocytes were observed.

A significant leukopenia was observed after a single oral dose (35.5 and 71 mg/kg) of TNB that was entirely due to decreased lymphocytes. Significant changes in the leukogram were not apparent during daily dosing up to four or 10 days. A decrease in serum triglyceride was observed in both the 4 and 10 day treatment periods (Chandra, 1995).

Gross Pathologic Lesions

The gross lesions noted with TNB toxicosis relate to the reticuloendothelial, reproductive and nervous systems. Common findings are splenomegaly, testicular atrophy, and hemorrhages in the brain. Animals that die acutely may have no gross pathologic changes other than chocolate-brown blood from severe methemoglobinemia.

Histopathologic Lesions

The microscopic lesions found in TNB toxicosis include significant changes in the spleen, kidneys, testicles, and brain. Consistent findings in the spleen are numerous erythropoietic foci throughout the red pulp and hemorrhage in a few lymphoid nodules around central arteries. Kidneys exhibit dose-related accumulation of hyaline droplets containing alpha-2u-globulin in the proximal tubules. Significant increases in cell proliferation within proximal tubules are also observed. Testicles have degenerating seminiferous tubules with numerous multinucleated giant cells, necrotic epithelium with cellular pyknosis and karyorrhexis, and a few mature well-differentiated spermatozoa. Sertoli cells are often markedly vacuolated or dead and dying with pyknosis and karyorrhexis. Brain lesions consist of malacia in the locus ceruleus and inferior colliculi along with the interpositus, lateral cerebellar, and superior olivary nuclei. Vacuolation of the neuropil and the presence of many glial cells characterize the malacia. Hemorrhage is often present in the previously mentioned brain nuclei as well as the locus ceruleus and inferior colliculi. Hemorrhages into the spaces of Virchow-Robin surrounding small

vessels with pronounced fibrinoid change in vessel walls are often observed (Chandra, 1995).

Neurotoxicosis

Many neurotoxicants exert their harmful effects directly on specific targets in the mammalian central or peripheral nervous system. Other chemicals may indirectly harm the nervous system by damaging other organs or affecting metabolic pathways that are important in the maintenance of nervous system homeostasis. The goal of scientific investigations is to outline the biochemical or molecular pathways of these neurotoxicants. Behavioral and clinical observations are also important to identify neurotoxicants that change nervous system function without causing morphologic change.

Classification of Neurotoxic Disease

Classifying neurotoxicants morphologically assumes that each toxicant has a primary focus of cellular damage (Spencer & Schaumburg, 1980). While this concept does not apply for all chemicals, it does allow for categorization of neurotoxicants. Several problems arise when trying to classify neurotoxicants morphologically. Morphologic changes may not occur with toxicants that are extremely potent and result in rapid death. Also, neurotransmission abnormalities may be expressed as impaired performance or behavioral changes on neurologic exams without concurrent pathology.

Functional observation batteries (FOBs) are tools employed by investigators and regulatory agencies to screen for potential neurotoxicants (Tilson, 1993). Groups of

neurologic tests are performed to evaluate compounds for onset, progression, duration, and reversibility of neurotoxic injury. These neurologic tests are another aid in the classification of neurotoxicants.

Morphologic classification of neurotoxicants involves determination of the site of damage to the nerve cell or its supporting structures. Toxicants may inflict damage by disruption of the nerve cell body (neuronopathies), axons (axonopathies), or supporting myelin sheath (myelinopathies). As stated earlier, neurotransmission interruption, blockage or accentuation may result in toxicity with no apparent structural damage to neurons or their supporting structures.

Neuronopathy involves insult to the nerve cell body (Thomas, 1980) and is usually widely distributed and irreversible (Anthony et al., 1996). However, insults to neurons with peculiar vulnerabilities in specific locations within the brain also occur, and may be expressed as changes in behavioral function or clinicals signs.

Axonopathies are neurotoxic disorders where the axon is the primary site of toxicity. Axonal degeneration is rapidly followed by loss of the myelin sheath, but the nerve cell body remains intact. Axons that are greatest in length are most susceptible to toxic insults. Axonal degeneration in the peripheral nervous system may allow for partial or complete recovery while damage to axons in the CNS is irreversible (Anthony et al., 1996).

Myelinopathies result from induced intramyelinic edema, the selective loss of myelin, termed demyelination (Anthony et al., 1996). Intramyelinic edema may be caused by changes at the transcript level of mRNA and if corrected early can be reversible (Veronesi et al., 1991). Segmental demyelination may develop as intramyelinic edema progresses or may also result from direct toxicity to the myelinating

cell. Schwann cells in the peripheral nervous system are often capable of remyelination while oligodendrocytes in the CNS have little regenerative capacity.

Structural similarity of many compounds with similar mechanisms of action at or around neuronal or neuromuscular synapses has led to their classification as neurotransmission toxicants. Toxicity resulting from these compounds is often considered reversible with time or may be counteracted by the use of appropriate antagonists (Anthony et al., 1996). However, if the toxic compound remains at the site of action for extended periods of time, the effects may be irreversible.

Possible Mechanisms of 1,3,5-trinitrobenzene - Induced Neurotoxicosis

Nitric Oxide - Mediated Vascular Effects and Neurotoxicosis

Nitric Oxide (NO) is a free radical that has been recognized as a major neuronal messenger and regulator of cerebral blood flow (Garthwaite et al., 1988; Iadecola et al., 1994). NO is not stored in the body but produced as needed by NO synthase (NOS). Endothelial cells, macrophages, and neurons have been documented to produce NO and L-citrulline from L-arginine and oxygen (Bredt, 1995).

NO has been linked with neurotoxicosis when produced in excess. Glutamate acting through N-methyl-D-aspartate (NMDA) receptors in the brain stimulates NO and reactive oxygen species (ROS) formation (Gunasekar et al., 1995). Excess production of NO and ROS from NMDA over-stimulation results in the death of neurons, probably from the generation of peroxynitrite. Blockage of NO formation by the removal of arginine from media, or presence of nitric oxide synthase (NOS) inhibitors blocks neuronal death (Bredt & Snyder, 1994). N^{G}-Nitro-L-arginine methyl ester (L-NAME) is

an inhibitor of NOS commonly used in studies on neuroeffector transmission (Rand & Li, 1995).

Vasodilation of blood vessels due to the generation of NO has been well documented (Iadecola et al., 1996). Activation of cerebellar parallel nerve fibers increases cerebral blood flow via production of neuronal NO. The relatively selective neuronal NOS inhibitor 7-nitroindazole (7-NI) has been used to block increased cerebral blood flow after stimulation of cerebellar parallel nerve fibers (Iadecola et al., 1996).

Changes in the cerebral vasculature have been implicated in the pathogenesis of 1, 3-DNB and other nitroaromatic-induced lesions. Previous literature was not found documenting the effects of NO on nitroaromatic-induced encephalopathy. NO may play a role in alteration of neurotransmission and/or the cerebral vasculature in TNB-induced neurotoxicosis.

Thiamine Deficiency

Thiamine is a coenzyme for the decarboxylation of pyruvate and the oxidation of alpha keto-glutamic acid. Thiamine deficiency can be induced experimentally with the thiamine antagonist pyrithiamine. Experimental thiamine deficiency is a classical model of metabolic encephalopathy resulting from low-grade oxidative deficit. Late stages of thiamine deficiency are characterized by hemorrhages in the brain stem and formation of large vacuoles within myelin sheaths. Studies utilizing immunoreactivity of IgG to assess the integrity of the BBB after pyrithiamine-induced thiamine deficiency have resulted in increased immunoreactivity in areas of the brain with histologic lesions (Calingasan et al., 1995). Brain immunohistochemistries have also detected

extravascular albumin leakage due to breakdown of the BBB after induced thiamine deficiency (Harata & Iwasaki, 1995). Increased levels of the excitatory amino acid glutamate have also been found in the thalami of rats with pyrithiamine-induced thiamine deficiency (Langlais & Zhang, 1993). The histologic lesions produced by 1,3-DNB and TNB resemble those described in mice and rats treated with pyrithiamine (Watanabe, 1978; Watanabe & Kanabe, 1978; Watanabe et al., 1981; Calingasan et al., 1995).

Oxidative Stress

Reactive oxygen species (ROS), such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist in all aerobic organisms. Molecular oxygen can readily react to form the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the most reactive species, the hydroxyl free radical ($HO^{\bullet}$). Excess production of reactive oxygen species can induce neurodegeneration due to damaged membranes (lipids), protein, and DNA. The brain is extremely vulnerable to attack from ROS due to a high concentration of unsaturated lipids, large iron stores, low metal binding capacity, low antioxidant levels and the inability to regenerate neurons (Acworth & Bailey, 1995).

The superoxide anion is formed when molecular oxygen acquires an additional electron. Superoxide radicals are produced when xanthine oxidase converts xanthine to uric acid. Auto-oxidation of hydroquinones, catecholamines and thiols also results in formation of superoxide (Acworth & Bailey, 1995). Phagocytic leukocytes produce superoxide and hydrogen peroxide as their major "microbicide" defense mechanism (Robinson & Badwey, 1995). Normally the super oxide anion is converted to hydrogen

peroxide by superoxide dismutase (SOD) in a process known as spontaneous dismutation. Altered SOD function has been linked with amyotrophic lateral sclerosis and Down's syndrome (Acworth & Bailey, 1995).

Several metabolic reactions including those catalyzed by SOD, D-amino acid oxidase and amine oxidase generate hydrogen peroxide. Hydrogen peroxide can also be produced in the breakdown of superoxide. Hydrogen peroxide can cross both the plasma and nuclear membranes contributing to DNA adduct formation. Enzymes such as catalase and peroxidases (eg. glutathione peroxidase) contribute to the breakdown of hydrogen peroxide (Acworth & Bailey, 1995).

Hydroxyl radicals are formed from either the superoxide anion (Haber-Weiss reaction) or from hydrogen peroxide (Fenton reaction). Both of these reactions require a transitional metal such as iron or copper. The toxicity of hydrogen peroxide and superoxide anion radicals may be due to their conversion to the hydroxyl free radical (Acworth & Bailey, 1995).

Peroxidation of membrane lipids by reactive oxygen species makes them more hydrophilic altering their normal structure. Cellular and nuclear membranes damaged by oxidative stress may have cell transport abnormalities or modified membrane bound receptors. Disruption of blood flow, ion transport, and synaptic transmission may result when arachidonic acid undergoes peroxidation by interfering with the production of prostaglandins, thromboxanes and leukotrienes (Acworth & Bailey, 1995).

Strong reducing agents like the nitroheterocyclic compounds are readily acted upon by nitroreductases, such as xanthine oxidase, aldehyde oxidase and microsomal NADPH-cytochrome c reductase. One proposed mechanism of 1,3-DNB-induced neurotoxicosis is the generation of oxygen reactive radicals. These radicals are

probably produced through futile redox cycling impairing mitochondrial function (Romero et al., 1995).

At least three endogenous mechanisms exist to prevent adverse sequelae from ROS. Enzymes, antioxidants, and metal chelators all aid in the breakdown, inactivation or prevention of harmful oxygen species.

Enzymatic removal of ROS may involve several enzymatic pathways and enzymes. Superoxide dismutase aids in the removal of superoxide and is located in mitochondria and the cytosol. Peroxisomes contain catalase that breaks down hydrogen peroxide to water and oxygen. Removal of organic hydroperoxides and hydrogen peroxide is accomplished in a reaction involving glutathione by glutathione peroxidase. Glutathione peroxidase also occurs in mitochondria and cytosol. Antioxidants are molecules that donate electrons to radical species to quench harmful chain reactions or neutralize ROS. Reduction of ROS leads to the antioxidant molecule being oxidized. After being oxidized, antioxidants require regeneration to continue their free radical scavenging and protectant capabilities. Many of the antioxidants in the organism act directly to reduce other oxidized antioxidants stripped of electrons in their protective roles. Many vitamins, phytochemicals, sulfhydryl compounds and uric acid are antioxidants that aid in the protection from ROS.

Vitamin E is a potent peroxyl radical scavenger, which terminates the chain reaction of lipid peroxidation. The antioxidant function of vitamin E is potentiated *in vitro* by other antioxidants such as vitamin C, α lipoic acid, and glutathione. Generation of radical species is also supported by research involving pretreatment of rats with large doses of vitamin E protecting against 1,3-DNB neurotoxicosis (Lowndes et al., 1994).

Binding of transitional metals such as iron and copper before they are able to generate free radicals is the protective capacity of metal chelation. Protein carriers like transferrin and lactoferrin keep iron bound as well as hemoglobin, myoglobin, and ferrodoxins. Copper is transported throughout the body by ceruloplasmin.

Depletion of Intracellular Glutathione

Glutathione and glutathione related enzyme systems play an important protective role against oxidative stress in the nervous system. Reduced glutathione in the central nervous system appears to be primarily localized in the neuropil and white matter tracts (Philbert et al., 1991). Depletion of mitochondrial glutathione has been shown to render astrocytes unable to combat oxidative stress and disrupt thiol dependent enzymatic pathways (Huang & Philbert, 1996). An *in vitro* study reported reduced glutathione levels decreased to about half those of controls when astrocytes were exposed to 0.5 mM 1,3-DNB over a 2 hour period (Romero et al., 1995). Studies have demonstrated that 1,3-DNB stimulates brain glucose utilization *in vivo* prior to vascular damage or lesion development (Romero et al., 1995). A hypothetical cause for this increased metabolic rate might involve a compensatory response for an increased demand of reducing equivalents [ie. glutathione (GSH), NADPH]. Generation of the free nitro radical reduction product has been shown to deplete cellular glutathione and NADPH as well as aid in production of the toxic superoxide radical (Romero et al., 1991).

Statement of Dissertation Problem and Hypotheses

Nitroaromatic compounds are commonly used in many military and industrial applications. Concern over release of these compounds into the environment has resulted in investigations to illuminate possible mammalian risk from exposure. The hematologic, reproductive and central nervous systems are generally affected by the harmful consequences of exposure to these compounds.

Oral gavage of 1,3,5-trinitrobenzene at 71 mg/kg once daily for 10 days in male Fischer-344 rats results in neurologic clinical signs as well as gross and histologic brain lesions (Chandra, 1995). Previously, my colleagues have described many of the clinical and pathological effects of TNB-induced neurotoxicosis. This dissertation is an attempt to investigate the pathophysiology underlying the clinical signs and pathology.

The purpose of the first part of this study is to examine the role of nitric oxide and thiamine in TNB-induced encephalopathy. These experiments will examine the effects of dosing agonists and antagonist of nitric oxide and thiamine while orally gavaging TNB (71 mg/kg) in male Fischer-344 rats once daily for 10 consecutive days. Nitric oxide has been shown to have many physiologic roles including regulating some aspects of vasopression and neurotransmission. The brain lesions induced by TNB have been described as having similar morphologic and histologic characteristics as thiamine induced encephalopathy. The hypothesis for this part of the study is that nitric oxide or thiamine plays a role in TNB-induced encephalopathy.

The second phase will examine parentally administered antioxidants (Vitamin E, Vitamin C, and N-acetylcysteine) in an attempt to modulate the severity of TNB toxicosis in Fischer-344 rats. The hypothesis of this study is that TNB-induced brain lesions may be prevented or decreased by exogenously administered antioxidants.

The next phase of this study will examine TNB dose-response *in vitro* studies. Astrocytes and endothelial cell *in vitro* cytotoxicity and metabolic activity will be assessed after exposure to TNB. These experiments will compare cytotoxicity of TNB with 1,3-DNB *in vitro* and determine if previous data from 1,3-DNB experimentation is reproducible. The hypothesis of this phase of the project is that TNB causes direct damage to astrocytes and endothelial cells in a similar fashion as 1,3-DNB.

Neurotoxicosis will be characterized using clinical signs and histopathology. Additionally, several indicators (body weight, organ weight, clinical pathology) of generalized toxic effects will further describe this syndrome. *In vitro* and *in vivo* data will be useful for a more complete description and future mechanistic studies. As depicted in Figure 5, below, the expression of this disease syndrome is multifactorial.

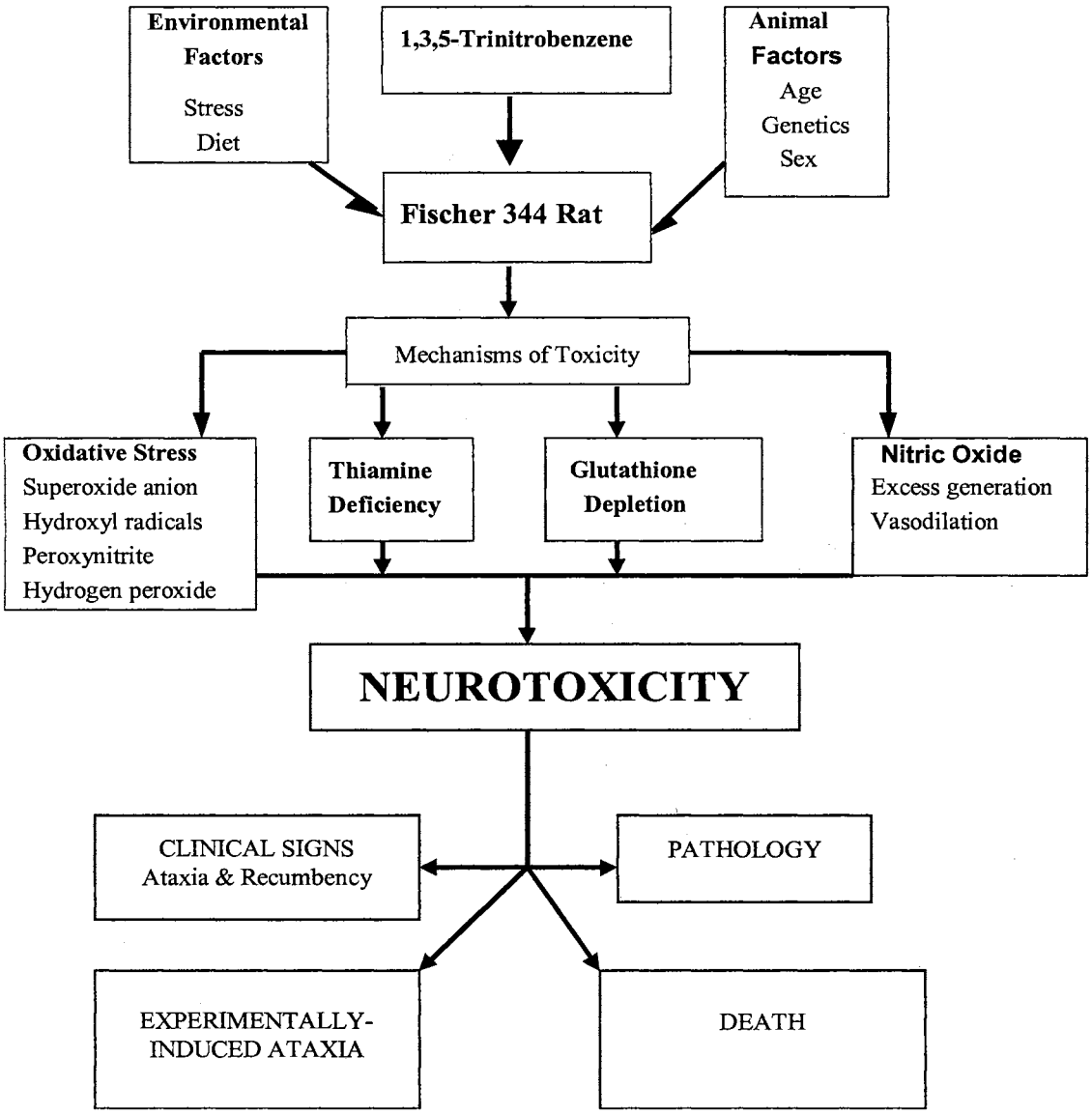

Figure 5. Potential mechanisms of the pathogenesis of TNB-induced neurotoxicosis.

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CHAPTER II

THE ROLE OF NITRIC OXIDE AND THIAMINE IN 1,3,5-TRINITROBENZENE-INDUCED NEUROTOXICOSIS

Abstract

1,3,5-Trinitrobenzene (TNB) is a munition chemical known to produce methemoglobinemia, hemolytic anemia, reproductive toxicity (testicular and epididymal degeneration) and gliovascular lesions in the brainstem of rats. The neurotoxic effects are similar in appearance and location to those produced by other nitroaromatic compounds and thiamine deficiency. To identify if nitric oxide (NO) or thiamine deficiency were involved in TNB induced toxicosis, *in vivo* experiments with compounds that either increased or decreased NO or thiamine were conducted in Fischer-344 rats gavaged with TNB at 71 mg/kg for 10 days. To determine the role of NO, L-arginine, the NO precursor, was administered intraperitoneally (IP) at 300 mg/kg 30 minutes prior to TNB treatment. N^G-Nitro-L-arginine methyl ester (L-NAME), the inhibitor of NO, was administered via drinking water at a concentration of 0.1 mg/ml for 10 days. The rats also received L-NAME intraperitoneally at 10 mg/kg 30 minutes prior to TNB treatment. To assess the role of thiamine, the rats were pretreated with pyrithiamine at 0.25 mg/kg, IP and thiamine at 100 mg/kg intramuscularly 30 minutes prior to TNB treatment.

The brains of all rats receiving TNB only had histological lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. Pretreatment with L-arginine did not alter the lesions induced by TNB except for no apparent pathology in the inferior colliculi. Similarly pretreatment with L-NAME did not induce changes in lesions in comparison with TNB administered alone. Rats treated with pyrithiamine and TNB had lesions consistent with rats dosed with TNB only. Thiamine supplementation along with TNB gavage did not prevent lesions similar in appearance to those induced by TNB only.

Oral administration of TNB induced an increase in whole blood thiamine levels compared to controls. However, co-administration of thiamine along with TNB produced a four-fold increase in whole blood thiamine levels compared with a two-fold increase when thiamine was administered alone. While a trend towards a decrease in whole blood thiamine levels was observed in rats dosed with pyrithiamine and TNB, and rats receiving pyrithiamine only, the decrease was not significant when compared with control animals. These data along with neuropathologic findings do not lend strong support to the hypothesis that nitric oxide or thiamine plays a significant role in TNB-induced neurotoxicosis.

Introduction

1,3,5-Trinitrobenzene (TNB) is a nitroaromatic compound produced during the manufacture of explosives (particularly trinitrotoluene) and other industrial chemicals. Concern over possible harmful physiological consequences from mammalian exposure

initiated research showing that exposure to TNB can result in detrimental hematologic, reproductive, and neurologic effects. The major difference between TNB-induced toxicosis and toxicosis induced by other nitroaromatics appears to be the time frame for lesions to appear and the quantity of compound administered to elicit similar toxicologic effects. It is currently unclear if these induced changes are due directly to TNB, it's metabolites, or other biochemical events initiated by TNB toxicosis.

Neurologic damage in Fischer-344 rats administered TNB at 71 mg/kg for 10 days included symmetrical brain stem lesions characterized by vascular damage including leakage of red blood cells into spaces of Virchow-Robin around arterioles (Chandra, 1995; Chandra et al., 1995).

The exact mechanisms for all of the neurotoxic consequences of TNB exposure are currently unknown. Therefore, nitric oxide, due to its role in vasopression and as a neurotransmitter was selected to investigate regarding any possible role in TNB toxicosis. Also, brain stem lesions induced by TNB had previously been described as having similarities in location and appearance of thiamine deficiency. Subsequently, thiamine deficiency was investigated as another possible sequelae following TNB induced toxicosis.

Nitric oxide (NO) is a free radical generated in biological systems. NO functions at low concentrations as a signal in many diverse physiological processes such as blood pressure regulation and at high concentrations as a defensive cytotoxin. NO has also been recognized as a major neuronal messenger and regulator of cerebral blood flow (Garthwaite et al., 1988; Iadecola et al., 1994). Association between NO produced in excess and neurotoxicosis has been documented (Eliasson et al., 1997; Leist et al.,

1997; Love, 1999; Pieper et al., 2000; Mandir et al., 2000). Neurotoxicosis may result from alterations in neurotransmission and/or changes in the cerebral vasculature.

Nitric oxide is derived from the terminal guanidino nitrogens of L-arginine via the action of nitric oxide synthase (NOS). In mammals, three distinct genes encode nitric oxide synthase (NOS) isozymes: neuronal (nNOS or NOS-1), cytokine-inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) (Marletta et al., 1988; Palmer et al., 1988; Knowles et al., 1989). NOS is soluble and responsible for NADPH, calcium and calmodulin-dependent (Bredt & Snyder, 1989) synthesis of citrulline and NO from L-arginine (Knowles et al., 1989). The NO formed by this reaction increases soluble guanylate cyclase in vascular smooth muscle and elevates cyclic GMP levels in arteries and veins (Ignarro et al., 1987; Bredt & Snyder, 1989; Knowles et al., 1989) resulting in relaxation of vascular smooth muscle and subsequent vasodilation. NO has been documented in the regulation of vascular and blood-brain barrier permeability (Janigro et al., 1994). NO has also been shown to modulate ion channels in excitable cells, thus affecting neuronal firing. Patch-clamp experiments display a modulatory action of NO as well as cGMP and cAMP on a hyperpolarization-activated current carried by both Na^+ and K^+ ions in blood-brain barrier endothelial cells (Janigro et al., 1994). In addition, NO is an important messenger molecule for various physiological responses that are implicated in the peripheral and the central nervous system (Nathan, 1992).

NO has been linked with neurotoxicosis when produced in excess. During cerebral ischemia, excessive production of the excitatory amino acid glutamate results in N-methyl-D-aspartate (NMDA) receptor overstimulation. NMDA overstimulation results in increased intracellular calcium levels which are responsible for excessive NO production. Excess production of NO from NMDA over-stimulation results in DNA

damage and over-activation of Poly (ADP-ribose) Polymerase (PARP). Excessive activation of PARP completely depletes cellular energy stores, resulting in cell death (Eliasson et al., 1997; Leist et al., 1997; Love, 1999; Pieper et al., 2000; Mandir et al., 2000). Reperfusion of ischemic tissue can initiate events that cause the simultaneous production of NO and superoxide. Superoxide and NO can rapidly react intracellularly and in the vascular lumen to form peroxynitrite ($ONOO^-$). Peroxynitrite is a powerful oxidant that can initiate lipid peroxidation or react with transition metals to form a powerful nitrating agent with reactivity suggestive of the nitronium ion (NO_2^+) (Varner & Beckman, 1995). Blockage of NO formation by the removal of arginine from media, or presence of nitric oxide synthase (NOS) inhibitors can block neuronal death (Bredt & Snyder, 1994).

TNB induced brain lesions have also been described as having similar morphologic and histologic characteristics as thiamine induced encephalopathy (Chandra, 1995; Chandra et al., 1995).

Thiamine consists of a molecule of pyrimidine and a molecule of thiazole linked by a methylene bridge. The principle function of thiamine in all cells is as the coenzyme cocarboxylase or thiamine pyrophosphate (TPP). Thiamine is the coenzyme for all enzymatic decarboxylations of α-keto acids. Thus it functions in the oxidative decarboxylation of pyruvate to acetate, which in turn is combined with coenzyme A for entrance into the tricarboxylic acid cycle (McDowell, 1989). In mammals, thiamine is essential in two oxidative decarboxylation reactions in the citric acid cycle. One of these reactions takes place in the mitochondria and the other reaction occurs in the cytoplasm. These are essential reactions for utilization of carbohydrates to provide energy. Thiamine deficiency in cultured glial cells impairs their ability to synthesize fatty

acids and cholesterol. The defect is related to reduced formation of key lipogenic enzymes (McDowell, 1989).

Substances with anti-thiamine activity are fairly common in nature and include structurally similar antagonists as well as structure-altering antagonists. The synthetic compound pyrithiamine chiefly blocks the esterification of thiamine with phosphoric acid, resulting in inhibition of the thiamine coenzyme cocarboxylase (McDowell, 1989). Late stages of thiamine deficiency are characterized by hemorrhages in the brain stem and formation of large vacuoles within myelin sheaths.

Studies concerning the role of NO and thiamine in TNB-induced neurotoxicosis are very limited. Therefore, the major objective of this study is to determine the role of both NO and thiamine in TNB-induced neurotoxicosis.

Materials and Methods

Animals

Young adult male, Fischer-344 rats weighing approximately 200 grams each were acquired from a commercial source (Charles River Laboratories Inc., Wilmington, MA). All rats were acclimatized for one week prior to experimentation and allowed free access to water and a standard commercial diet (Purina Rat Chow). Rats were housed individually in plastic cages with stainless steel wire covers and bedded on Sani Chips (P.J. Murphy Forest Products, Rochelle Park, NJ). All rat cages were kept in the same room maintained at a temperature of 21° - 24 °C with a twelve-hour light-dark cycle. All rats were housed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985).

Drugs

1,3,5-trinitrobenzene (99.83% purity) was obtained from Naval Surface Warfare Center and the purity of the compound was confirmed by HPLC. TNB to be gavaged was prepared fresh daily by adding to corn oil (25 mg/ml) and mixing with a Potter-Elvehjem tissue grinder. L-arginine (Sigma, St. Louis, MO), a nitric oxide precursor was solubilized in normal saline (300 mg/ml) for IP injection. The nitric oxide synthase (NOS) inhibitor N^G-Nitro-L-arginine methyl ester (Sigma, St. Louis, MO) was solubilized in drinking water (0.1 mg/ml) for consumption or normal saline (10 mg/ml) for IP injection depending on which respective group was to receive the drug. The thiamine inhibitor pyrithiamine (Sigma, St. Louis, MO) was solubilized in normal saline (0.5 mg/ml) for IP injection. Thiamine hydrochloride (200 mg/ml) (Phoenix Pharmaceutical, St. Joseph, MO) was used for IP injection.

Experimental Protocol: Roles of Nitric Oxide and Thiamine

Experiment # 1: Determination of the Role of Nitric Oxide

Thirty-five rats were randomly divided into 7 treatment groups consisting of five animals each. Group 1 served as the control. Rats in this group received corn oil (the vehicle) per os. Group 2 rats were gavaged with TNB at 71 mg/kg. Group 3 rats received the nitric oxide precursor L-Arginine at 300 mg/kg IP 30 minutes before gavage of TNB at 71 mg/kg. Group 4 rats received the NOS inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME) in their drinking water at 0.1 mg/ml. Fresh water was replaced with L-NAME treated water for group 4 rats 24 hours before the first gavage dose of TNB at 71 mg/kg. Group 4 rats had their water replaced every 24 hours with fresh water

containing L-NAME and were allowed free access to the treated water. Group 5 rats received L-NAME at 10 mg/kg IP 30 minutes before gavage of TNB at 71 mg/kg. Group 6 rats received L-Arginine at 300 mg/kg IP 30 minutes before gavage of corn oil. Group 7 rats received L-NAME at 10 mg/kg IP 30 minutes before gavage of corn oil. Treatment of the rats with TNB at 71 mg/kg and respective inducers and inhibitors of nitric oxide were continued once daily for 10 consecutive days. Rat groups 6 and 7 received treatment with corn oil once daily for 10 consecutive days. All rats excluding Group 4 consumed tap water ad libitum during the experimental period. Daily water consumption was monitored for each rat to obtain an estimation of L-NAME treated water (Group 4) vs. tap water only (all other groups) consumption. Rats were individually caged to obtain a more accurate calculation of L-NAME and water consumption.

Experiment # 2: Determination of the Role of Thiamine

A second experiment was conducted to assess the role of thiamine deficiency in the induced encephalopathy. Twenty-five rats were divided into 5 groups consisting of five animals each. The control rats from the 1st experiment (Role of Nitric Oxide) were used as the control animals in the 2nd experiment. Group 1 received TNB at 71 mg/kg per os. Group 2 rats received the thiamine inhibitor pyrithiamine at 0.25 mg/kg IP before gavage of TNB at 71 mg/kg. Group 3 received thiamine hydrochloride at 100 mg/kg IM 30 minutes before administration of TNB at 71 mg/kg per os. Group 4 rats received pyrithiamine at 0.25 mg/kg IP before gavage of corn oil (the vehicle) per os. Group 5 rats received thiamine hydrochloride at 100 mg/kg IM 30 minutes before administration of corn oil. Treatment of the rats with TNB at 71 mg/kg was continued

once daily for 10 consecutive days. Rat groups 4 and 5 received treatment with corn oil once daily for 10 consecutive days. Water consumption of rats in each group was monitored daily. Treatment of the rats with TNB at 71 mg/kg and respective inducers and inhibitors of thiamine was continued once daily for 10 consecutive days.

Clinical Assessment of Neurotoxicosis

Water consumption was measured daily for the entire experimental period. Water bottles were weighed daily (at approximately the same time) and refilled with fresh water.

All rats were assessed at least twice daily for clinical signs of ataxia. All rats were examined walking after each daily treatment and at other times not associated with dosing. Forced ambulation allowed detection of slightly ataxic rats.

Morphologic and Histopathologic Examination

At the end of each experimental periods (24 hours after day 10) all the rats were anesthetized using CO_2 thereby inducing a surgical plane of anesthesia. Under anesthesia, rats were euthanized by exsanguination. Rats that died or became critically ill before the end of the experimental period were handled similar to those that reached the end of the experiment. At necropsy, tissues from the brain were collected for histopathology. Tissues were fixed in a phosphate buffered, 10% formaldehyde solution and kept cool until processing. Following fixation, the tissues were processed in a series of alcohol dehydration steps and embedded in paraffin. Tissues in paraffin blocks were sectioned into 4 – 6 μm slices, placed on glass slides and stained with

hematoxylin and eosin. Step serial sections of the brain from the same morphologic locations in each rat were examined in all experimental groups. Morphologic assessment of the encephalopathy was used as the end point of neurotoxicosis. The histopathologic lesions were graded as: 0 no lesion; 1 mild changes involving only scattered areas; 2 moderate changes, with many vacuolated areas; 3 severe changes involving hemorrhage, vacuolation and gliosis. The presence or absence of lesions were noted within each brain nuclei for rats within different treatment groups and presented in tabular form.

Determination of Thiamine Levels

Under anesthesia, rats were euthanized by exsanguination. Whole blood was collected and frozen before shipment for thiamine analysis. Blood was assayed for thiamine in treatment groups 1 (Experiment # 1) and 1, 2, 3, 4 and 5 from experiment # 2. Thiamine levels were obtained from Colorado Veterinary Diagnostic Laboratories, Colorado State University, Fort Collins, CO using a microbiologic assay (Icke & Nicol, 1994).

Data Analysis

All data were initially examined by analysis of variance (ANOVA) with a general linear model (GLM) using SAS (SAS Institute, Carey, NC). Mean ± standard deviation of whole blood thiamine levels and water consumption for each group were calculated and compared to appropriate controls using Dunnett's test. Differences were considered significant at $P< 0.05$.

Results

Water Consumption

An increase (3 – 40 %) in water consumption although not statistically different was observed in all groups treated either with TNB alone, TNB & L-NAME$_{(water)}$, TNB & L-NAME$_{(ip)}$, TNB & pyrithiamine, L-NAME only, L-arginine only, and pyrithiamine only except the group receiving TNB & L-arginine (Figure 1).

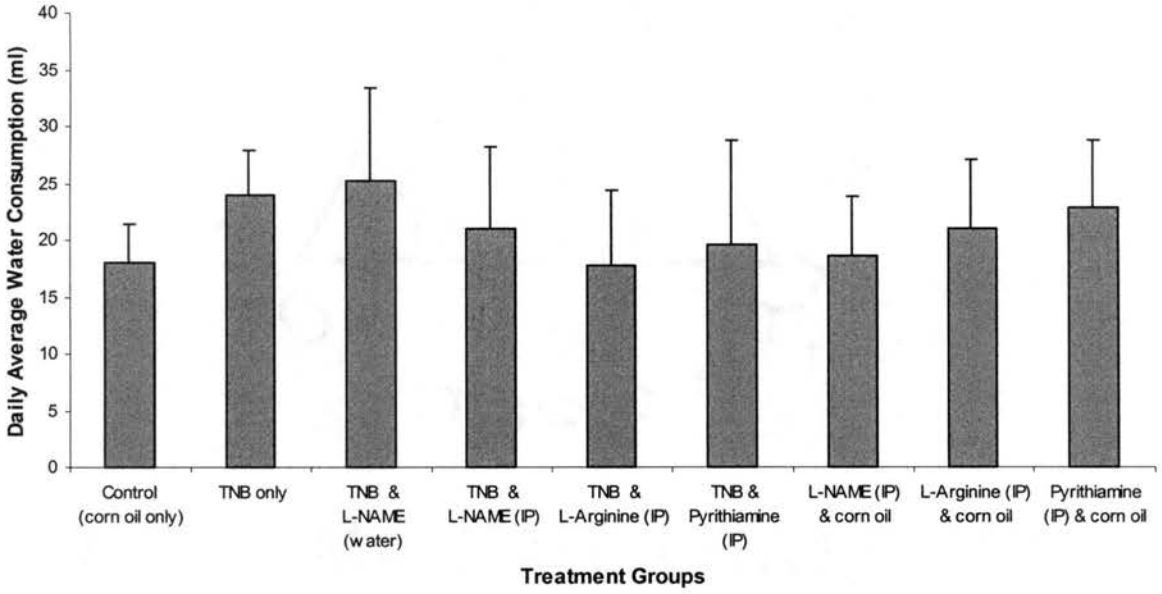

Figure 1. Average daily water consumption of male Fischer-344 rats administered 71 mg/kg TNB for 10 days. Results are expressed as means ± SD (*=Statistically Significant, $P < 0.05$).

Clinical Assessment of Neurotoxicosis

Oral administration of TNB at 71 mg/kg for 10 days appeared to induce a transient (3 – 4 hours) depression. This depression was characterized by inactivity with the rats lying in a sternal position. During this depressive state, the rats exhibited clinical signs of hypernea along with cyanosis of the tail, feet and ears. Clinical signs were apparent 30 minutes to 1 hour after dosing. These clinical signs were consistent with the severe methemoglobinemia induced by TNB that has been previously described by Chandra et al (Chandra, 1995; Chandra et al., 1995). TNB with co-administration of L-arginine, L-NAME, pyrithiamine or thiamine resulted in similar clinical signs to those observed with rats treated with TNB alone.

By the 5th day of the experiment, all rats receiving TNB only became extremely hyperactive immediately following treatment. These rats ran continuously from one end of the cage to the other and buried their heads or entire bodies underneath the cage bedding. These hyperactive episodes usually lasted less than 5 minutes with the rats then entering the depressed state described above. This post-treatment hyperactive or excitement phase was observed each day after dosing for the remainder of the experiment. TNB with co-administration of L-arginine, L-NAME, pyrithiamine or thiamine resulted in similar clinical signs to those observed with rats treated with TNB alone after the 5th day of the experiment.

Rats receiving TNB and the NO precursor L-arginine had varying degrees of ataxia by the 6th day of the experiment. By the 8th day of the experiment, all rats receiving TNB with or without inducers or inhibitors of nitric oxide or thiamine had some degree of neurologic abnormality.

Morphologic and Histopathologic Evaluation of the Brain

Macroscopically, all rats dosed with 71 mg/kg TNB (including animals receiving inducers or inhibitors of nitric oxide or thiamine) had petechial hemorrhages around the cerebellar peduncles and brain stem. On histologic examination of the brain, rats within the control (vehicle only) group had neither gross nor microscopic lesions in the brain (Figure 2). The brains of all rats receiving TNB only had histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei on the medulla and cerebellum. Pretreatment with L-arginine did not alter the lesions induced by TNB except for no detectable lesions in the inferior colliculi. Similarly, pretreatment with L-NAME (either in the water or administered IP) did not induce changes in lesions in comparison with TNB administered alone. Rats treated with pyrithiamine and TNB had lesions consistent with rats dosed with TNB only. Thiamine supplementation along with TNB gavage did not prevent lesions similar in appearance to those induced by TNB only.

The inferior colliculi were affected bilaterally in all rats receiving TNB except the group administered L-Arginine IP 30 minutes before dosing. Lesions were also observed in the superior olivary nuclei, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum (Table I). The lesions in the medulla and cerebellum were observed in all groups receiving TNB with or without inducers or inhibitors of nitric oxide or thiamine.

Lesions of the inferior colliculi were characterized by a vascular response consisting of swollen endothelial cells and breakdown of the tunica media to form a fibrinoid material plus cellular debris that was both karyorrhectic and pyknotic. The perivascular neuropil was vacuolated and there was an influx of astroglia at the periphery. Frequently, hemorrhage was present in spaces of Virchow-Robin. In neurons adjacent to the inferior colliculi, there was central chromatolysis.

In the area of the superior olivary nuclei (Figure 3) there were areas of hemorrhage bilaterally and vacuolation (malacia) of the neuropil. Often, vessels in these nuclei had a fibrinoid change extending into the neuropil. The vascular endothelium was swollen with central chromatolysis of neurons surrounding these vessels. Perivascular astroglial accumulations and malacia were also present.

The interpositus nuclei, lateral cerebellar nuclei and superior cerebellar peduncles had hemorrhage and vacuolation (malacia). There was central chromatolysis of neurons in and surrounding these nuclei. Vascular endothelium was swollen with perivascular edema and fibrinoid change. There was a cellular influx of glial cells in and around these nuclei.

Lesions of the inferior colliculi as described above were observed consistently in each study group receiving TNB except the rats dosed with L-Arginine and TNB. The other regions of the brain consistently affected in animals receiving TNB were the superior olivary nuclei, interpositus nuclei, lateral cerebellar nuclei and the superior cerebellar peduncles. Rats receiving L-arginine, L-NAME, pyrithiamine, or thiamine along with TNB, all had lesions in these same brain nuclei. However, not all of these nuclei were uniformly involved in each rat. All of the rats with brain lesions had some degree of neurologic signs.

TABLE I.

SUMMARY OF BRAIN LESIONS FROM FISCHER-344 MALE RATS GAVAGED WITH 1,3,5-TRINITROBENZENE (71 mg/kg) FOR 10 DAYS

Treatment Group	Inferior Colliculi	Superior Olive Nuclei	Superior Cerebellar Peduncles	Interpositus Nuclei	Lateral Cerebellar Nuclei
Control (corn oil only)	0/5	0/5	0/5	0/5	0/5
TNB only	5/5	4/5	1/5	2/5	2/5
TNB & L-NAME$_{(water)}$	4/4	4/4	1/4	4/4	4/4
TNB & L-NAME$_{(ip)}$	3/5	5/5	1/5	2/5	2/5
TNB & L-Arginine	0/5	3/5	1/5	2/5	2/5
TNB & Pyrithiamine	1/5	3/5	1/5	3/5	3/5
TNB & Thiamine	5/5	4/5	3/5	4/5	4/5
L-NAME Control	0/5	0/5	0/5	0/5	0/5
L-Arginine Control	0/5	0/5	0/5	0/5	0/5
Pyrithiamine Control	0/5	0/5	0/5	0/5	0/5
Thiamine Control	0/5	0/5	0/5	0/5	0/5

Legend for Treatments in 10-day trial:

L-NAME$_{(water)}$ = N^G-Nitro-L-arginine methyl ester 0.1 mg/ml added to drinking water

L-NAME$_{(ip)}$ = N^G-Nitro-L-arginine methyl ester (IP) 10 mg/kg administered 30 minutes before TNB

L-Arginine = L-Arginine (IP) 10 gm/kg administered 30 minutes before TNB

Pyrithiamine = Pyrithiamine (IP) 0.25 mg/kg administered 30 minutes before TNB

Thiamine = Thiamine (IP) 100 mg/kg administered 30 minutes before TNB

Controls = Administered same dosages as treatment groups excluding TNB

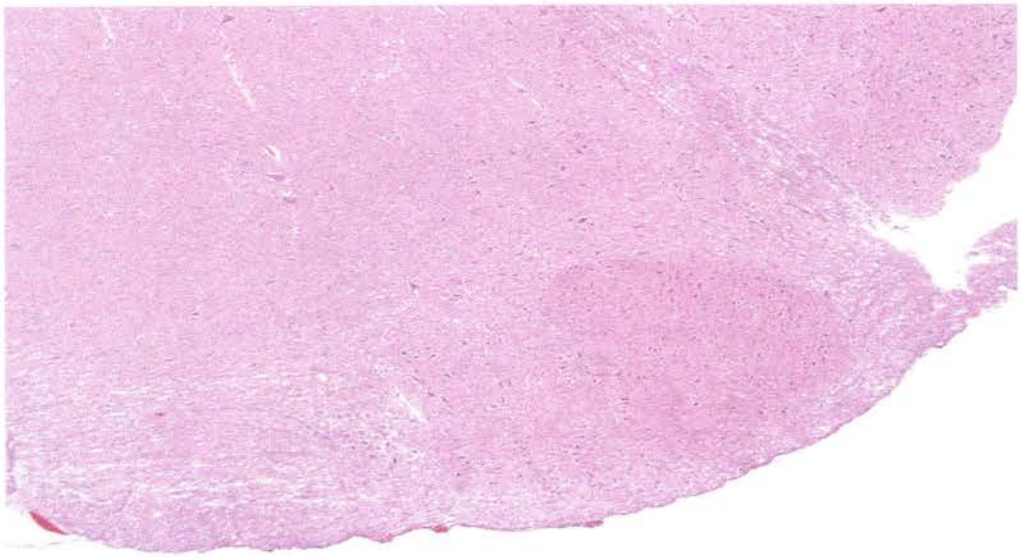

Figure 2. Photomicrograph of the superior olivary nucleus from a control Fischer-344 rat. No microscopic lesions are observed. H & E. 20X

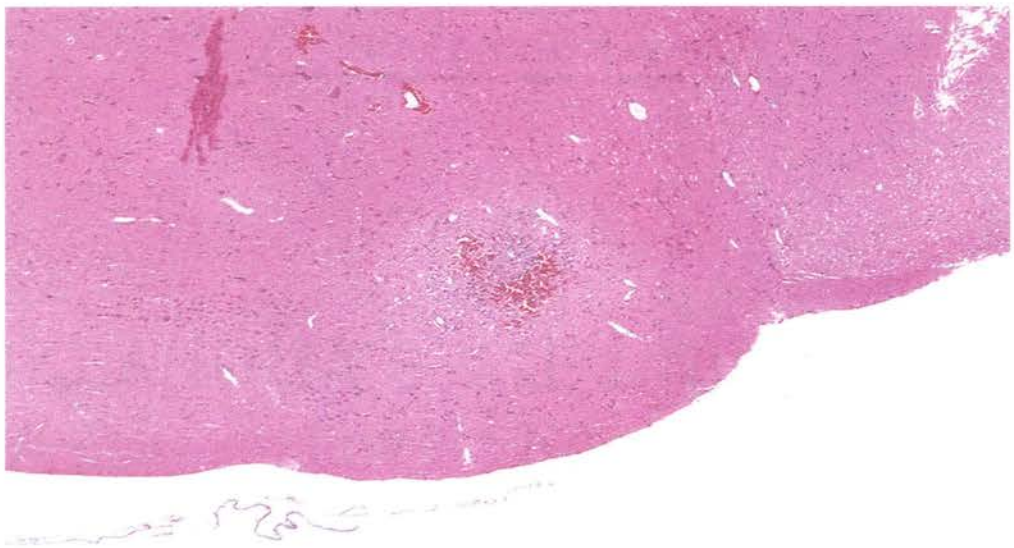

Figure 3. Photomicrograph of the superior olivary nucleus from a Fischer-344 rat treated with TNB at 71 mg/kg for 10 days. Note the focus of hemorrhage and gliosis within the neuropil. H & E. 20X

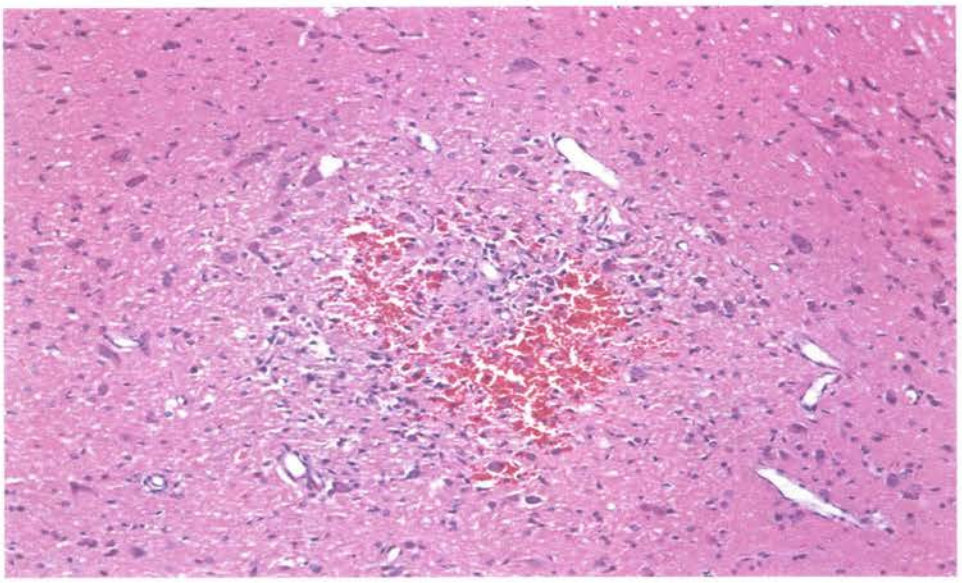

Figure 4. Higher magnification of the superior olivary nucleus from the Fischer-344 rat treated with TNB at 71 mg/kg for 10 days (figure 4). Note the areas of hemorrhage and accumulation of microglial cells. H & E. 100X

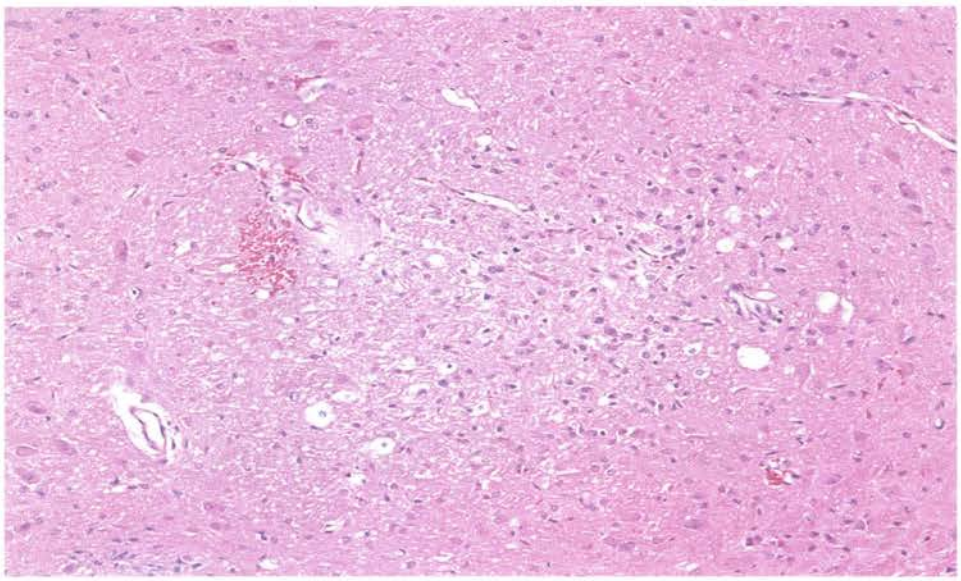

Figure 5. Photomicrograph of the superior olivary nucleus from a Fischer-344 rat treated with N^G-Nitro-L-arginine methyl ester (10 mg/kg IP) 30 minutes before gavage of TNB at 71 mg/kg for 10 days. Note the focus of hemorrhage, vacuolation and gliosis within the neuropil. H & E. 100X

Determination of Whole Blood Thiamine Levels

Whole blood thiamine levels displayed an increasing trend in rats receiving TNB only (360 μg/L) versus control rats (254 μg/L). Whole blood thiamine levels were significantly increased in all groups receiving thiamine IP (133% in thiamine only [593 μg/L] and 251% in rats receiving TNB & thiamine [890 μg/L]). While pyrithiamine alone (206 μg/L) and pyrithiamine pre-treatment before TNB gavage (105 μg/L) both induced a decrease in whole blood thiamine, the differences were not statistically significantly from control rats (Figure 6).

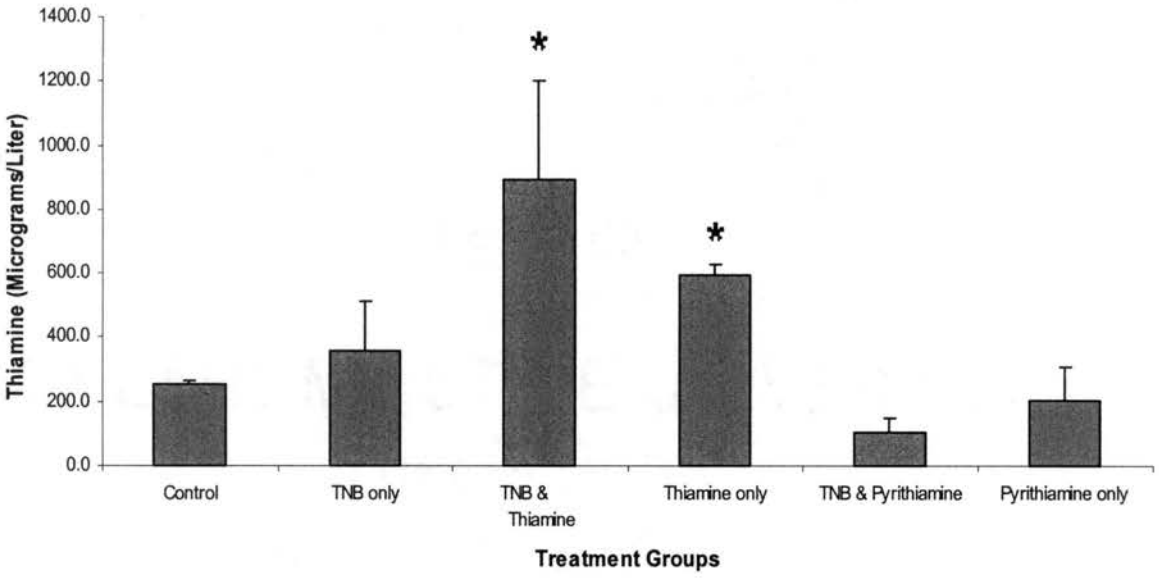

Figure 6. Whole blood thiamine levels of male Fischer-344 rats administered 71 mg/kg TNB for 10 days. Results are expressed as means ± SD (*=Statistically Significant, $P < 0.05$).

Discussion

A trend towards increased water consumption was observed among all groups of rats treated with TNB only, TNB plus either L-NAME in water or with L-NAME administered intraperitoneally, and TNB with pyrithiamine except the group receiving combination of both TNB & L-arginine. A similar trend was noticed in those rats that were not treated with TNB but received L-NAME, L-arginine, or pyrithiamine. However, none of these changes were statistically significant when compared with control rats that received the vehicle (corn oil) only. These data indicate that the rats that had L-NAME in their water supply (0.1 mg/ml) consumed an equivalent dose of L-NAME as those rats that received L-NAME (10 mg/kg) by IP injection.

All rats receiving TNB only became extremely hyperactive by the 5th day of the experiment immediately following oral administration. These rats ran continuously from one end of the cage to the other and buried their heads or entire bodies underneath the cage bedding. These hyperactive episodes usually lasted less than 5 minutes with the rats then entering the depressed state accompanied by the severe methemoglobinemia. This post-treatment hyperactive or excitement phase was observed each day after dosing for the remainder of the experiment. TNB with co-administration of L-arginine, L-NAME, pyrithiamine or thiamine resulted in similar clinical signs to those observed with rats treated with TNB alone after day 5 of the experiment. Rats receiving TNB and the NO precursor L-arginine had varying degrees of ataxia on the 6th day of the experiment. On the 8th day of the experiment, all rats receiving TNB with or without inducers or inhibitors of nitric oxide or thiamine had some degree of neurologic abnormality that included either extreme ataxia or recumbency. Recumbent rats were unable to

ambulate even when prodded by hand. The observed clinical signs appear to be the sequelae to the histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem.

Control animals receiving corn oil only for 10 days had no clinical or histological evidence of neuropathology. The brains of all rats receiving TNB only had histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. These findings are in agreement with those previously reported (Chandra, 1995; Chandra et al., 1995; Chandra et al., 1999).

The exact mechanisms for all of the neurotoxic consequences of TNB exposure are currently unknown. Therefore, nitric oxide, due to its role in vasopression and as a neurotransmitter was selected to investigate regarding any possible role in TNB-induced toxicosis.

L-arginine, the immediate precursor of NO was selected as a specific drug to utilize in evaluating the role of NO in TNB induced toxicosis. L-arginine is converted to NO and citrulline by nitric oxide synthase (Palmer et al., 1988; Bredt & Snyder, 1989; Knowles et al., 1989; Vallance et al., 1989). L-arginine has been shown to promote NO-dependent vasodilation, increase regional cerebral blood flow, and reduce infarction volume in the rat (Morikawa et al., 1994). N^G-Nitro-L-arginine methyl ester (L-NAME) was chosen as the nitric oxide synthase inhibitor due to its nonselective action on all isoforms of NOS (Vaupel et al., 1995; Lissbrant et al., 1997; Kurihara et al., 1998).

The findings that prior administration of L-arginine did not alter brain lesions induced by TNB except in the inferior colliculi strongly suggest that there might not be any significant alteration in the endogenous NO levels in the brain.

Several studies have presented data supporting a role for intracollicular NO in the processing and transmission of the acoustic input to the auditory cortex in the rat (Grassi et al., 1995; Iannone et al., 1996). Other investigators have suggested that brain nuclei exhibiting increased neuronal activity may subsequently display an increased neuronal activity may subsequently display an increase in severity of lesions observed with dinitrobenzene toxicosis (Ray et al., 1992; Holton et al., 1997). L-arginine may decrease metabolic activity in the inferior colliculi to prevent subsequent hemorrhage and vacuolation induced by TNB.

Changes in both morphologic and histopathologic lesions induced by oral administration of L-NAME prior to TNB were not significantly different from those of TNB treated rats. These results suggest that NO might not be implicated in the pathogenesis of TNB-induced neurotoxicosis.

Only one dosage level of L-arginine and L-NAME were used throughout the experiments (Ashwal et al., 1995; Rachmilewitz et al., 1995; Zhang et al., 1995; Kamii et al., 1996). Perhaps, future studies using multiple and increasing dosages of L-arginine and L-NAME with simultaneous determination of endogenous NO levels in different regions of the brain should yield different results supporting the role of NO in TNB-induced neurotoxicosis.

Attempts to manipulate endogenous nitric oxide in the central nervous system either by supplementing rats with the precursor or inhibiting nitric oxide synthase did not yield fruitful directions concerning the role of nitric oxide in TNB-induced neurotoxicosis.

Any further experimental approaches to determine whether or not NO plays a significant role in TNB-induced encephalopathy should include mechanisms to accurately assess NO levels concomitantly with observed clinical signs and histopathologic changes in the affected brain nuclei.

Substantial evidence indicates that TNB-induced brain lesions have also been described as having similar morphologic and histologic characteristics as thiamine induced encephalopathy (Chandra, 1995; Chandra et al. 1995).

The dose of pyrithiamine, a known thiamine antagonist, used in this study did not alter the normal morphologic and histologic features of the brain stem and cerebellum. This can be explained by the fact that adequate induction of thiamine deficiency in rodents dosed with pyrithiamine requires placing them on a thiamine deficient diet (Hakim et al., 1983). Rats treated with pyrithiamine and TNB had lesions consistent with those of rats dosed with TNB only. Thiamine supplementation failed to prevent TNB-induced neuropathologic changes.

Oral administration of TNB induced an increase in whole blood thiamine levels compared to controls. However, co-administration of thiamine along with TNB produced a four-fold increase in whole blood thiamine levels compared with a two-fold increase when thiamine was administered alone. While a trend towards a decrease in whole blood thiamine levels was observed in rats dosed with pyrithiamine and TNB, and rats receiving pyrithiamine only, the decrease was not significant when compared with control animals. Interestingly, results presented in this study indicate that oral administration of TNB alone could induce an increase in whole blood levels of thiamine. These data along with neuropathologic findings do not lend support to the hypothesis that thiamine plays a significant role in TNB-induced neurotoxicosis.

The findings on morphologic and histologic lesions of the brain induced-by TNB are corroborated by previous work by (Chandra, 1995; Chandra et al., 1995; Chandra et al., 1999). All rats in every group that received TNB at 71 mg/kg for 10 days had brain lesions (35/35). Areas of brain damage are consistently associated with or surrounding vessels (primarily arterioles) (Chandra et al., 1999). Damage to the vascular endothelium may result in secondary neuronal damage, either by allowing TNB or it's metabolites entry past the blood brain barrier (BBB) or leakage of vascular components injurious to neuronal tissue. Other investigators (Romero et al., 1991, 1995) have suggested the vascular bed plays a role in the pathogenesis of DNB neurotoxicosis.

Since the neuropathologic changes are remarkably similar in location and appearance when comparing DNB and TNB, future studies should concentrate on the vascular lesions. Antioxidants have been used *in vitro* to reduce cytotoxicity to endothelial cells exposed to DNB (Romero et al., 1996). Intracellular acidosis leading to free radical formation, lipid peroxidation and mitochondrial dysfunction, have been implicated in increased cytotoxicity. Oxidative stress could further be suggested to disrupt endothelial cellular interactions with astrocytes compromising the blood brain barrier (Romero et al., 1996). Future studies in elucidating the potential pathophysiologic mechanism of TNB-induced neurotoxicosis should include the administration of agents protecting the vascular endothelium from oxidative stress and free radical damage.

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CHAPTER III

THE ROLE OF OXIDATIVE STRESS IN 1,3,5-TRINITROBENZENE-INDUCED NEUROTOXICOSIS

Abstract

1,3,5-Trinitrobenzene (TNB) is a munition chemical known to produce methemoglobinemia, hemolytic anemia, reproductive toxicity (testicular and epididymal degeneration) and gliovascular lesions in the brainstem of rats. The neurotoxic effects are similar in appearance and location to those produced by other nitroaromatic compounds and thiamine deficiency. To identify if reactive oxygen species (ROS) were involved in TNB induced toxicosis, *in vivo* experimentation with antioxidant compounds such as vitamin E, vitamin C, and N-acetylcysteine were performed in Fischer-344 rats gavaged with TNB at 71 mg/kg for 10 days. To determine the role of vitamin E, *dl* α-tocopheryl acetate, was administered intramuscularly (IM) at 200 mg/kg 30 minutes prior to TNB treatment for 10 days. Vitamin C (ascorbic acid) was administered subcutaneously (SQ) at 200 mg/kg 30 minutes before TNB treatment for 10 days. The rats also received N-acetylcysteine, the glutathione precursor, intraperitoneally (IP) at 140 mg/kg as a loading dose 30 minutes prior to TNB treatment. After administration of the loading dose, N-acetylcysteine was continually administered intraperitoneally at 70 mg/kg every 6 hours for 10 days. To assess the role of antioxidant combinations, the rats were pretreated with *dl* α-tocopheryl acetate along with N-acetylcysteine or vitamin

C plus N-acetylcysteine following the same administration routes and dosing schedules for single antioxidant groups prior to TNB treatment.

The brains of all rats receiving TNB only had histological lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. Pretreatment with vitamin E (*dl* α-tocopheryl acetate) did not induce changes in lesions in comparison with TNB administered alone. Rats treated with vitamin C and TNB had lesions consistent with rats dosed with TNB only. N-acetylcysteine (NAC) supplementation along with TNB gavage did not prevent lesions similar in appearance to those induced by TNB only. Similarly, combination of NAC with either vitamin E or vitamin C did not induce changes in lesions when compared with TNB administered alone.

Histopathologic findings of the brain do not lend strong support to the hypothesis that exogenously administered vitamin E, vitamin C and/or N-acetylcysteine offer protection against TNB-induced neurotoxicosis.

Introduction

1,3,5-Trinitrobenzene (TNB) is a nitroaromatic compound produced during the manufacture of explosives (particularly trinitrotoluene) and other industrial chemicals. Concern over possible harmful physiological consequences from mammalian exposure initiated research proving that ingested TNB can result in encephalopathy.

Neurologic damage in Fischer-344 rats administered TNB at 71 mg/kg for 10 days is characterized by vascular damage including leakage of red blood cells into spaces of

Virchow-Robin around arterioles in the brain stem (Chandra, 1995; Chandra et al., 1995). Intracellular acidosis leading to free radical formation, lipid peroxidation and mitochondrial dysfunction, have been implicated in neurotoxicosis associated with 1,3-dinitrobenzene exposure. Oxidative stress could further be suggested to disrupt endothelial cellular interactions with astrocytes compromising the blood brain barrier (Romero et al., 1996).

Reactive oxygen species (ROS) are constantly formed in the mammalian body and removed by antioxidant defenses. Reactive oxygen species such as free radicals and peroxides, are derived from the metabolism of oxygen and can be responsible for neurodegeneration arising from an imbalance between radical-generating and radical-scavenging systems. Molecular oxygen can readily react to form the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the most reactive species, the hydroxyl free radical ($HO^{\bullet}$). Neurodegeneration may be induced from oxidative stress due to damaged membranes (lipids), protein, and DNA. This results in DNA modification and oxidation of fats. Superoxide and the hydroxyl free radical initiate lipid peroxidation in the cellular, mitochondrial, nuclear, and endoplasmic reticulum membranes. Increased cell permeability from damaged membranes induces Ca^{++} influx that further damages mitochondrial integrity. Cysteine sulfhydryl groups and other amino acid residues on proteins are oxidized and degraded. Nuclear and mitochondrial DNA are oxidized, resulting in strand breaks and other types of damage. The brain is highly susceptible to oxidative stress due to it's high concentration of unsaturated lipids, large iron stores, low metal binding capacity, low antioxidant levels and the inability to regenerate neurons (Acworth & Bailey, 1995).

The exact mechanisms for all the neurotoxic consequences of TNB exposure are currently unknown. Therefore, vitamin E and vitamin C, due to their roles as radical-scavenging antioxidants were selected to investigate regarding any neuroprotective effects on TNB-induced toxicosis. N-acetylcysteine (NAC), a precursor of reduced glutathione (GSH), also promotes detoxification by acting directly as a free radical scavenger. N-acetylcysteine was additionally selected for study as a means of preventing GSH depletion and preventing neuropathology from oxidative stress.

Vitamin E (tocopherol) is a lipid soluble free radical scavenger and primarily functions to protect against lipid peroxidation of membranes. Vitamin E is the only antioxidant vitamin whose sole physiological role is to quench free radical reactions (Marks et al., 1996). At least one important function of Vitamin E is to interrupt production of free radicals at their initial stage. This antioxidant also ensures erythrocyte stability and maintenance of capillary blood vessel integrity (McDowell, 1989). Attached to a highly hydrophobic hydrocarbon portion of membranes or plasma lipoproteins, α-tocopherol has a hydroxyl group whose hydrogen atom is easily removed. Hence, peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with α-tocopherol instead of adjacent fatty acid side chains (Hailliwell, 1991). This terminates the chain reaction of lipid peroxidation. Some thiol compounds, such as reduced GSH, might also be involved in regenerating α-tocopherol from its radical form *in vivo* (Wefers & Sies, 1988).

Vitamin C occurs in two forms, namely the reduced ascorbic acid and the oxidized dehydroascorbic acid. Only the L isomer of ascorbic acid has activity. Although the majority of the vitamin exists as ascorbic acid, both forms are biologically active. Vitamin C is the least stable, and therefore most easily destroyed, of all vitamins

(McDowell, 1989). The most clearly established functional role for vitamin C involves collagen biosynthesis. Inadequate levels of vitamin C result in impairment of collagen synthesis due to lowered ability to hydroxylate lysine and proline. Vitamin C can react with the vitamin E radical to regenerate vitamin E into its natural antioxidant form. In this process, vitamin C becomes a free radical (the semiascorbyl radical). The vitamin C radical can be regenerated in turn by thiols such as GSH.

Reduced glutathione (GSH) is the cell's primary preventive antioxidant. When GSH neutralizes harmful oxidative radicals, it becomes oxidized in such a manner that two reduced glutathione molecules are joined together via a disulfide bond.

N-acetylcysteine (NAC) is a precursor of GSH that relies on the sulfhydryl group for its antioxidant properties. The sulfhydryl (SH) group is responsible for a great deal of the metabolic activity of NAC, while the acetyl-substituted amino group makes the molecule more stable against oxidation. As a source of sulfhydryl groups, NAC can stimulate GSH synthesis, enhance glutathione-S-transferase activity, promote detoxification, and act directly on reactive oxidant radicals (De Vries & De Flora, 1993). Evidence, both *in vitro* and *in vivo*, indicates NAC is able to enhance the intracellular biosynthesis of GSH. In cell culture experiments, NAC promotes the uptake of cystine from the culture medium for cellular GSH biosynthesis (Issels et al., 1988). *In vivo*, NAC has also been shown to increase intracellular levels of GSH after experimental depletion (Nakata K et al., 1996). NAC exerts a protective effect against paraquat-induced cytotoxicity by acting as a GSH precursor and by enhancing intracellular concentrations of GSH (Hoffer E et al., 1996). NAC also has a protective effect on acetaminophen toxicosis because of its ability to act as a precursor of intracellular GSH (Corcoran & Wong, 1986). Sulfhydryl groups are essential for defense against reactive oxygen

species. NAC is a powerful scavenger of hypochlorous acid, and is capable of reducing hydroxyl radicals and hydrogen peroxide (Aruoma et al., 1989). In animal experiments, NAC has been shown to be protective against oxygen toxicity to the lung caused by prolonged administration of 100 percent oxygen (Wagner et al. 1989). Regarding its ability to protect against neurotoxicosis, pharmacokinetic information is not available as to whether or not N-acetylcysteine crosses the blood-brain barrier (Holdiness, 1991). Administration of GSH depletors such as diethyl maleate (DEM) to adult male rats was found to decrease brain and liver GSH and increase the blood brain barrier (BBB) permeability to micromolecular tracers (sodium fluorescein and [14C]sucrose) in a dose-dependent manner. Treatment with N-acetylcysteine and GSH provided a partial to full protection against DEM-induced brain GSH depletion and BBB dysfunction; however, the treatment with α-tocopherol or ascorbic acid were not effective (Agarwal & Shukla, 1999).

Studies concerning the role of antioxidants, particularly vitamin E, vitamin C, and NAC in TNB-induced neurotoxicosis are very limited. Therefore, the major objective of this study is to determine the role of vitamin E, vitamin C, and N-acetylcysteine in TNB-induced neurotoxicosis.

Materials and Methods

Animals

Young adult male, Fischer-344 rats weighing approximately 200 grams each were acquired from a commercial source (Charles River Laboratories Inc., Wilmington, MA). All rats were acclimatized for one week prior to experimentation and allowed free access

to water and a standard commercial diet (Purina Rat Chow). Rats were housed individually in plastic cages with stainless steel wire covers and bedded on Sani Chips (P.J. Murphy Forest Products, Rochelle Park, NJ). All rat cages were kept in the same room maintained at a temperature of 21° - 24 °C with a twelve-hour light-dark cycle. All rats were housed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985).

Drugs

1,3,5-trinitrobenzene (99.83% purity) was obtained from Naval Surface Warfare Center and the purity of the compound was confirmed by HPLC. TNB to be gavaged was prepared fresh daily by adding to corn oil (25 mg/ml) and mixing with a Potter-Elvehjem tissue grinder. Vitamin E (*dl* α-tocopheryl acetate) (950 mg/ml) (Sigma, St. Louis, MO) was used for IM injection. Ascorbic acid (vitamin C) (500 mg/ml) (Luitpold Pharmaceuticals, Shirley, NY) was used for SQ injection. Acetylcysteine (Mucosil^{TM}) (200 mg/ml) (Dey, Napa, CA) was used for IP injection.

Experimental Protocol: Roles of Vitamin E, Vitamin C, and N-acetylcysteine

Sixty rats were randomly divided into 12 treatment groups consisting of five animals each. Group 1 served as the control. Rats in this group received corn oil (the vehicle) per os. Group 2 rats were gavaged with TNB at 71 mg/kg. Group 3 rats received vitamin E at 200 mg/kg IM 30 minutes before gavage of TNB at 71 mg/kg. Group 4 rats received vitamin C at 200 mg/kg SQ 30 minutes before gavage of TNB at

71 mg/kg. Group 5 rats received a loading dose of N-acetylcysteine at 140 mg/kg IP 30 minutes before the 1st gavage dose of TNB at 71 mg/kg. N-acetylcysteine was continuously administered throughout the remainder of the experiment at 70 mg/kg q 6 hours until experimental termination. Group 6 rats received vitamin E and N-acetylcysteine in combination (at the same dosage levels for both drugs as Group 3 and Group 5) 30 minutes before gavage of TNB at 71 mg/kg. Group 7 rats received vitamin C and N-acetylcysteine in combination (at the same dosage levels for both drugs as Group 4 and Group 5) 30 minutes before gavage of TNB at 71 mg/kg. Treatment of the rats with TNB at 71 mg/kg and respective vitamin E or vitamin C was continued once daily for 10 consecutive days. Group 8 rats received Vitamin E at 200 mg/kg IM 30 minutes before gavage of corn oil. Group 9 rats received Vitamin C at 200 mg/kg SQ 30 minutes before gavage of corn oil. Group 10 rats received a loading dose of N-acetylcysteine at 140 mg/kg IP 30 minutes before the 1st gavage dose of corn oil. N-acetylcysteine was continuously administered throughout the remainder of the experiment at 70 mg/kg q 6 hours until experimental termination. Group 11 rats received vitamin E and N-acetylcysteine in combination (at the same dosage levels for both drugs as Group 8 and Group 10) 30 minutes before gavage of corn oil. Group 12 rats received vitamin C and N-acetylcysteine in combination (at the same dosage levels for both drugs as Group 9 and Group 10) 30 minutes before gavage of corn oil. Rat groups 8, 9, 10, 11, and 12 received treatment with corn oil once daily for 10 consecutive days. All rats consumed tap water ad libitum during the experimental period.

Clinical Assessment of Neurotoxicosis

All rats were assessed at least every 6 hours for clinical signs of ataxia. All rats were examined walking after each daily TNB dosing and at other times not associated with n-acetylcysteine administration (every 6 hours). Forced ambulation allowed detection of slightly ataxic rats.

Morphologic and Histopathologic Examination

At the end of the experimental period (24 hours after day 10) all the rats were anesthetized using CO_2 thereby inducing a surgical plane of anesthesia. Under anesthesia, rats were euthanized by exsanguination. Rats that died or became critically ill before the end of the experimental period were handled similar to those that reached the end of the experiment. At necropsy, tissues from the brain were collected for histopathology. Tissues were fixed in a phosphate buffered, 10% formaldehyde solution and kept cool until processing. Following fixation, the tissues were processed in a series of alcohol dehydration steps and embedded in paraffin. Tissues in paraffin blocks were sectioned into 4 – 6 μm slices, placed on glass slides and stained with hematoxylin and eosin. Step serial sections of the brain from the same morphologic locations in each rat were examined in all experimental groups. Morphologic assessment of the encephalopathy was used as the end point of neurotoxicosis. The histopathologic lesions were graded as: 0 no lesion; 1 mild changes involving only scattered areas; 2 moderate changes, with many vacuolated areas; 3 severe changes involving hemorrhage, vacuolation and gliosis. The presence or absence of lesions

were noted within each brain nuclei for rats within different treatment groups and presented in tabular form.

Results

Clinical Assessment of Neurotoxicosis

Oral administration of TNB at 71 mg/kg for 10 days appeared to induce a transient (3 – 4 hours) depression. This depression was characterized by inactivity with the rats lying in a sternal position. During this depressive state, the rats exhibited clinical signs of hypernea along with cyanosis of the tail, feet and ears. Clinical signs were apparent 30 minutes to 1 hour after dosing. These clinical signs were consistent with the severe induced methemoglobinemia from TNB that has been previously described by Chandra et al (Chandra, 1995; Chandra et al., 1995). TNB with co-administration of vitamin E, vitamin C, NAC, vitamin E plus NAC or vitamin C plus NAC resulted in clinical signs similar to those observed with rats treated with TNB alone.

By the 5th day of the experiment, all rats receiving TNB only became extremely hyperactive immediately following treatment. These rats ran continuously from one end of the cage to the other and buried their heads or entire bodies underneath the cage bedding. These hyperactive episodes usually lasted less than 5 minutes with the rats then entering the depressed state described above. This post-treatment hyperactive or excitement phase was observed each day after dosing for the remainder of the experiment. TNB with co-administration of vitamin E, vitamin C, NAC, vitamin E plus NAC or vitamin C plus NAC resulted in clinical signs similar to those observed with rats treated with TNB alone after the 5th day of the experiment.

Rats receiving TNB along with vitamin C and N-acetylcysteine had varying degrees of ataxia by the 4th day of the experiment. Rats receiving TNB only and rats dosed with TNB and vitamin C in combination had mild to moderate ataxia by the 5th day of the experiment. By the 7th day of the experiment, all rats receiving TNB with or without combinations of vitamin E, vitamin C and/or N-acetylcysteine had some degree of neurologic abnormality.

Macroscopically, all rats dosed with 71 mg/kg TNB had petechial hemorrhages around the cerebellar peduncles and brain stem. Histologically, the inferior colliculi were affected bilaterally in all rats receiving TNB. Lesions were also observed in the superior olivary nuclei, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. The lesions in the medulla and cerebellum were observed in all groups receiving TNB.

Morphologic and Histopathologic Evaluation of the Brain

Macroscopically, all rats dosed with 71 mg/kg TNB (including animals receiving vitamin E, vitamin C, N-acetylcysteine, vitamin E & N-acetylcysteine, or vitamin C & N-acetylcysteine) had petechial hemorrhages around the cerebellar peduncles and brain stem. On histologic examination of the brain, rats within the control (vehicle only) group had neither gross nor microscopic lesions in the brain (Figure 1). The brains of all rats receiving TNB only had histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei on the medulla and cerebellum.

Pretreatment with vitamin E, vitamin C, N-acetylcysteine, vitamin E plus N-acetylcysteine, and vitamin C plus N-acetylcysteine did not alter the lesions induced by TNB.

The inferior colliculi were affected bilaterally in all rats receiving TNB. Lesions were also observed in the superior olivary nuclei, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum (Table I). The lesions in the medulla and cerebellum were observed in all groups receiving TNB with or without pretreatment with antioxidant drugs (vitamin E, Vitamin C, N-acetylcysteine, vitamin E plus N-acetylcysteine, and vitamin C plus N-acetylcysteine).

Lesions of the inferior colliculi were characterized by a vascular response consisting of swollen endothelial cells and breakdown of the tunica media to form a fibrinoid material plus cellular debris that was both karyorrhectic and pyknotic. The perivascular neuropil was vacuolated and there was an influx of astroglia at the periphery. Frequently, hemorrhage was present in spaces of Virchow-Robin. In neurons adjacent to the inferior colliculi, there was central chromatolysis.

In the area of the superior olivary nuclei there were areas of hemorrhage bilaterally and vacuolation (malacia) of the neuropil. Often, vessels in these nuclei had a fibrinoid change extending into the neuropil. The vascular endothelium was swollen with central chromatolysis of neurons surrounding these vessels. Perivascular astroglial accumulations and malacia were also present.

The interpositus nuclei (Figures 2, 3, and 4), lateral cerebellar nuclei and superior cerebellar peduncles had hemorrhage and vacuolation (malacia). There was central chromatolysis of neurons in and surrounding these nuclei. Vascular endothelium was

swollen with perivascular edema and fibrinoid change. There was a cellular influx of glial cells in and around these nuclei.

Lesions of the inferior colliculi as described above were observed consistently in each study group receiving TNB with or without concurrent antioxidant treatment. The other regions of the brain consistently affected in animals receiving TNB were the superior olivary nuclei, interpositus nuclei, lateral cerebellar nuclei and the superior cerebellar peduncles. Rats receiving vitamin E, Vitamin C, N-acetylcysteine, vitamin E plus N-acetylcysteine, and vitamin C plus N-acetylcysteine along with TNB, all had lesions in these same brain nuclei. However, not all of these nuclei were uniformly involved in each rat. All of the rats with brain lesions had some degree of neurologic signs.

TABLE I.

SUMMARY OF BRAIN LESIONS FROM FISCHER-344 MALE RATS GAVAGED WITH 1,3,5-TRINITROBENZENE (71 mg/kg) FOR 10 DAYS

Treatment Group	Inferior Colliculi	Superior Olive Nuclei	Superior Cerebellar Peduncles	Interpositus Nuclei	Lateral Cerebellar Nuclei
Control (corn oil only)	0/5	0/5	0/5	0/5	0/5
TNB only	5/5	5/5	4/5	4/5	5/5
TNB & Vitamin E	4/5	4/5	5/5	4/5	4/5
TNB & Vitamin C	5/5	5/5	4/5	5/5	5/5
TNB & AC	4/4	4/4	4/4	4/4	4/4
TNB & Vit. E & AC	5/5	5/5	5/5	5/5	4/5
TNB & Vit. C & AC	5/5	4/5	3/5	4/5	4/5
Vitamin E Control	0/5	0/5	0/5	0/5	0/5
Vitamin C Control	0/5	0/5	0/5	0/5	0/5
AC Control	0/5	0/5	0/5	0/5	0/5
Vit. E & AC Control	0/5	0/5	0/5	0/5	0/5
Vit. C & AC Control	0/5	0/5	0/5	0/5	0/5

Legend for Treatments in 10-day trial:

Vitamin E = *dl* α-tocopheryl acetate (IM) 200 mg/kg administered 30 minutes before TNB

Vitamin C = ascorbic acid (SQ) 200 mg/kg administered 30 minutes before TNB

AC = N-acetylcysteine (IP) 140 mg/kg [Loading dose], 70 mg/kg q 6 hours for 10 days

Controls = Administered same dosages as treatment groups excluding TNB

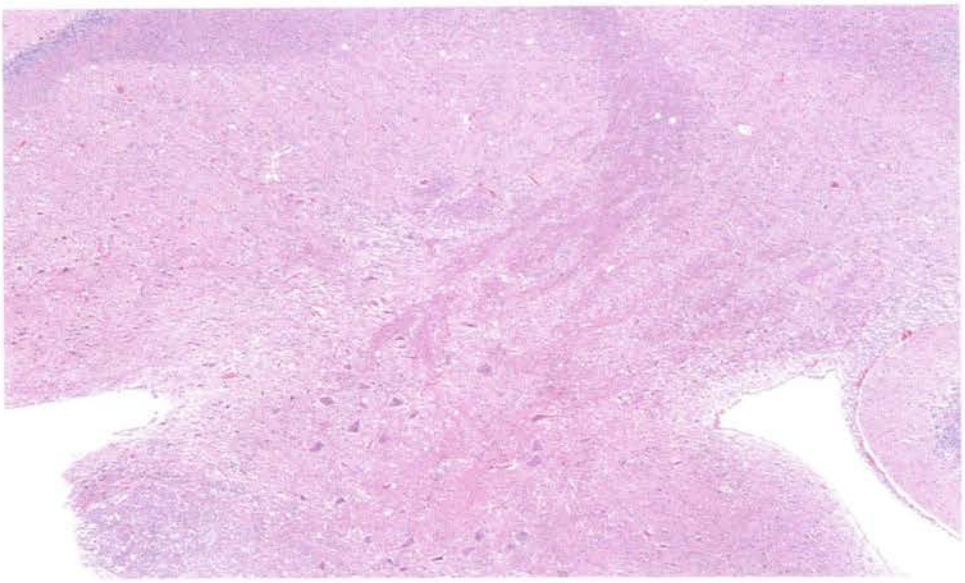

Figure 1. Photomicrograph of the interpositus nucleus from a control Fischer-344 rat. No microscopic lesions are observed. H & E. 20X

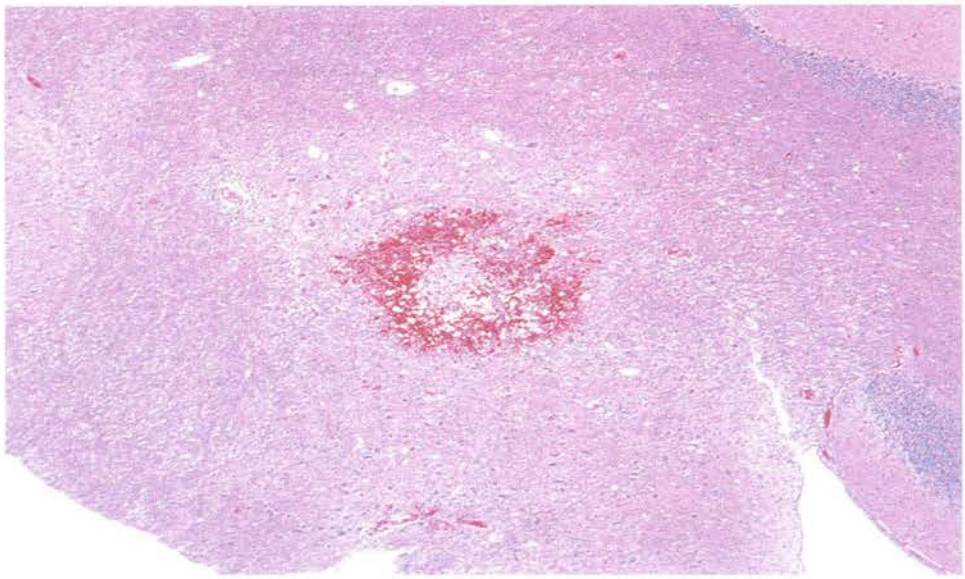

Figure 2. Photomicrograph of the interpositus nucleus from a Fischer-344 rat pretreated with vitamin C (200 mg/kg SQ) 30 minutes prior to administration of TNB at 71 mg/kg for 10 days. Note the focus of hemorrhage and malacia within the neuropil. H & E. 20X

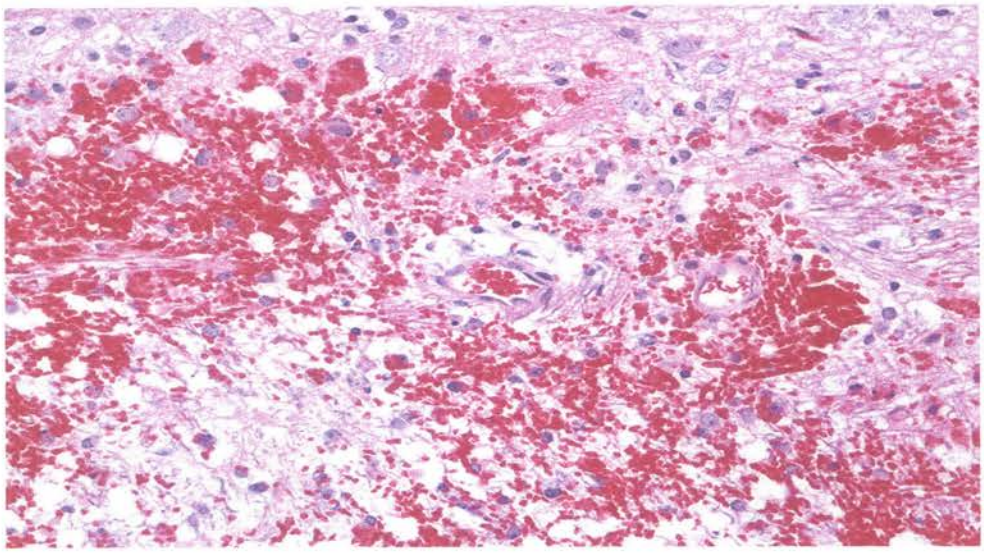

Figure 3. Higher magnification of the interpositus nucleus from the Fischer-344 rat treated with vitamin C (200 mg/kg SQ) 30 minutes prior to administration of TNB at 71 mg/kg for 10 days. Note the swollen endothelial cells accompanied by areas of hemorrhage and malacia. H & E. 200X

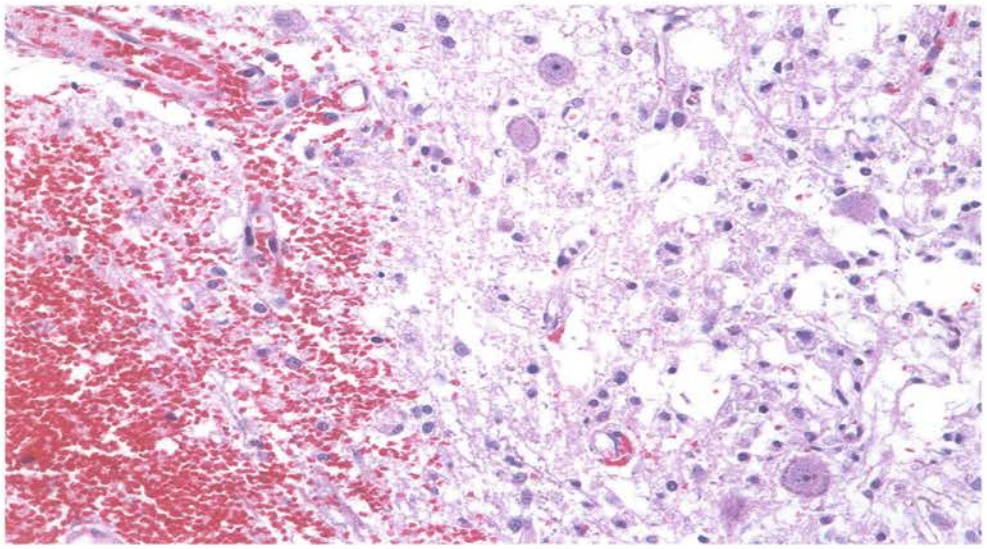

Figure 4. Photomicrograph of the interpositus nucleus from a Fischer-344 rat treated with vitamin E (200 mg/kg IM) 30 minutes prior to administration of TNB at 71 mg/kg for 10 days. Note the areas of hemorrhage and malacia. H & E. 200X

Discussion

All rats receiving TNB only became extremely hyperactive by the 5th day of the experiment immediately following oral administration. These rats ran continuously from one end of the cage to the other and buried their heads or entire bodies underneath the cage bedding. These hyperactive episodes usually lasted less than 5 minutes with the rats then entering the depressed state accompanied by the severe methemoglobinemia. This post-treatment hyperactive or excitement phase was observed each day after dosing for the remainder of the experiment. TNB with co-administration of vitamin E, vitamin C, NAC, vitamin E plus NAC or vitamin C plus NAC resulted in similar clinical signs as those observed with rat treated with TNB alone after the 5th day of the experiment. Rats receiving TNB with co-administration of vitamin C plus N-acetylcysteine had varying degrees of ataxia by the 4th day of the experiment. Rats receiving TNB only and rats dosed with TNB and vitamin C in combination had mild to moderate ataxia by the 5th day of the experiment. By the 7th day of the experiment, all rats receiving TNB with or without combinations of vitamin E, vitamin C and/or N-acetylcysteine displayed either extreme ataxia or recumbency. Recumbent rats were unable to ambulate even when prodded by hand. Similar to the previous experiment involving inducers or inhibitors of nitric oxide and thiamine, the observed clinical signs appear to be the result of histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem.

Control animals receiving corn oil only for 10 days had no clinical signs or histologic evidence of neuropathology. The brains of all rats receiving TNB only had histologic lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary

nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. These findings are very similar and in total agreement with those previously reported (Chandra et al., 1996).

Vitamin E, a lipid soluble free radical scavenger, was utilized in an attempt to prevent the neuropathologic consequences of TNB-induced toxicosis. The primary function of vitamin E is prevention of lipid peroxidation of membranes. When reactive oxygen species initiate damage via lipid peroxidation without being subsequently scavenged, a second free radical is generated which can react with a second macromolecule to continue a chain reaction. Vitamin E protects against lipid peroxidation by donating a hydrogen atom that scavenges the radical containing the free electron therefore ending the chain reaction (Hailliwell, 1991).

Changes in both morphologic and histopathologic lesions induced by intramuscular administration of vitamin E prior to TNB were not significantly different from those of TNB treated rats. These results suggest that vitamin E by itself may not provide adequate protection against TNB-induced neurotoxicosis.

Only one dosage level of vitamin E was used throughout the experiment. Also, biochemical measurement for generated lipid peroxidation products or vitamin E levels in affected brain nuclei were not performed. Perhaps, future studies using multiple and increasing dosages of vitamin E with simultaneous determination of lipid peroxidation products along with tissue vitamin E levels will yield different results supporting the role of vitamin E in protection against TNB-induced neurotoxicosis.

Vitamin C, due to its antioxidant properties was also selected as a drug to utilize in an attempt to prevent brain stem lesions induced by TNB. Vitamin C has also been shown to react with the vitamin E radical to regenerate vitamin E into its natural

antioxidant form. Vitamin C has also been shown to decrease lipid peroxidation in the brain induced from iron leaking from disrupted tissue (Rauhala & Chiueh, 2000). Supplementation with vitamin C has also reduced lipid peroxidation products in the liver and enhanced the activities of scavenging enzymes along with elevation of vitamin E levels in response to ethanol induced oxidative stress in guinea pigs (Suresh et al., 1999).

Changes in both morphologic and histopathologic lesions induced by subcutaneous administration of vitamin C prior to TNB were not significantly different from those of TNB treated rats. These results suggest that vitamin C alone may not provide adequate protection against TNB-induced neurotoxicosis.

Similar to vitamin E, only one dosage level of vitamin C was used throughout the experiment. Also, biochemical measurement for generated lipid peroxidation products or ascorbic acid levels in affected brain nuclei were not performed. Perhaps, future studies using multiple and increasing dosages of vitamin C along with simultaneous determination of lipid peroxidation products and analysis of tissue vitamin C levels will yield different results supporting the role of vitamin C in protection against TNB-induced neurotoxicosis.

N-acetylcysteine (NAC), a known glutathione precursor, has been used experimentally to combat neuronal injury originating from oxidative stress (Xiong et al., 1999; Fontaine et al., 2000; Pocernich et al., 2000). Treatment with NAC, has also provided a partial to full protection against diethyl maleate-induced brain (microvessel) GSH depletion and blood brain barrier dysfunction (Agarwal & Shukla, 1999). Therefore, NAC was selected as a drug to utilize in an attempt to prevent the brain stem lesions induced by TNB. Co-administration of vitamin E or vitamin C with NAC was also

used in an attempt to ameliorate or diminish the neuropathologic consequences of oral TNB exposure.

Changes in both morphologic and histopathologic lesions induced by intraperitoneal administration of NAC prior to TNB were not significantly different from those of TNB treated rats. Combination treatments using vitamin E or vitamin C with NAC also did not alter the lesions induced by rats receiving TNB only. These results suggest that NAC either alone or in combination with vitamin E or vitamin C may not provide adequate protection against TNB-induced neurotoxicosis.

Attempts to determine the role of oxidative stress in the central nervous system by providing exogenous antioxidants (vitamin E and vitamin C) either alone or in combination with a precursor (NAC) of an endogenous antioxidant did not yield fruitful directions concerning the role of oxidative stress in TNB-induced neurotoxicosis. Any further experimental attempts to determine whether or not oxidative stress plays a significant role in TNB-induced encephalopathy should include analyzing for the numerous metabolic products generated during lipid peroxidation. Assays for aldehydes (thiobarbituric acid assays), more specific but less sensitive assays (ultraviolet absorbance by conjugated dienes) or specific, highly sensitive assays that require expensive instrumentation (mass spectral analysis of hydroxy fatty acids). A sensitive and specific colorimetric assay based on the measurement of malondialdehyde and 4-hydroxyalkenals is a frequent compromise (Esterbauer & Cheeseman, 1990).

Another factor to consider when evaluating the success of experiments to prevent TNB-induced neurotoxicosis is the way the antioxidants were administered. Perhaps microinfusion of the antioxidants directly into the brain nuclei to prevent uptake by other body organs would have yielded significantly different results.

Rats are able to synthesize ascorbic acid without its supplementation and may not be a suitable animal species to use in determining whether or not vitamin C plays a role in prevention of oxidative stress induced by TNB. The guinea pig is often used as an experimental animal when vitamin C deficiencies are to be reproduced. Assuming that the brain lesions can be replicated in guinea pigs, using them as an animal model might reveal more definitive data regarding protection from exogenously administered vitamin C (Suresh et al., 1999).

Depletion of glutathione has been demonstrated to increase rat brain susceptibility to the gliovascular lesions caused by another nitroaromatic compound, dinitrobenzene (Hu et al., 1999). Analysis of glutathione levels within the affected brain nuclei should also explain whether or not glutathione depletion in a major factor in TNB-induced encephalopathy. Also, experiments with agents that deplete glutathione from the brain, such as buthionine sulfoximine (BSO), may aid in explaining the role of glutathione in preventing TNB-induced lesions.

Free radical or oxidative injury may be a fundamental mechanism underlying the neurologic pathology induced by TNB and prevention of lesions may still lie in therapy using free radical scavengers (antioxidants) to prevent, delay, or ameliorate this neurologic disorder. However, the biochemistry of oxidative pathobiology is complex, and optimum antioxidant therapeutic options may vary and need to be tailored to this individual neurologic toxicosis. The results of preventing other ROS toxicoses using various antioxidants, including vitamin E, tirilazad, N-acetylcysteine, α-lipoic acid, and ebselen, have been mixed (Delanty & Dichter, 2000). Potential reasons for these mixed results include lack of pretrial dose-finding studies and failure to appreciate and characterize the individual unique oxidative processes occurring in different toxicoses.

Moreover, therapy with antioxidants may need to be initiated earlier in acute neurologic disorders to achieve an appreciable clinical benefit.

The findings on morphologic and histologic lesions of the brain induced-by TNB are corroborated by previous work by (Chandra, 1995; Chandra et al., 1995; Chandra et al., 1999). All rats in every group that received TNB at 71 mg/kg for 10 days had brain lesions (29/29). Areas of brain damage are consistently associated with or surrounding vessels (primarily arterioles) (Chandra et al., 1999). Damage to the vascular endothelium may result in secondary neuronal damage, either by allowing TNB or it's metabolites entry past the blood brain barrier (BBB) or leakage of vascular components injurious to neuronal tissue. Other investigators (Romero et al., 1991, 1995) have suggested the vascular bed plays a role in the pathogenesis of DNB neurotoxicosis.

Since the neuropathologic changes are remarkably similar in location and appearance when comparing DNB and TNB, future studies should concentrate on the vascular lesions. Antioxidants have been used *in vitro* to reduce cytotoxicity to endothelial cells exposed to DNB (Romero et al., 1996). Intracellular acidosis leading to free radical formation, lipid peroxidation and mitochondrial dysfunction, have been implicated in increased cytotoxicity. Oxidative stress could further be suggested to disrupt endothelial cellular interactions with astrocytes compromising the blood brain barrier (Romero et al., 1996). Future studies in elucidating the potential pathophysiologic mechanism of TNB-induced neurotoxicosis should include *in vitro* studies with cellular components of the blood brain barrier (astrocytes and endothelial cells) to more accurately assess the exact mechanisms of the encephalopathy.

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CHAPTER IV

AN *IN VITRO* STUDY OF 1,3,5-TRINITROBENZENE TOXICITY ON TYPE I ASTROCYTES FROM FISCHER-344 RATS

Abstract

1,3,5-Trinitrobenzene (TNB) is an industrial chemical causing gliovascular lesions in the brain stem similar to those produced by thiamine deficiency and nitroaromatic compounds including *m*-dinitrobenzene. To identify early preneuropathic indices of toxicity, the effects of varying concentrations (0 – 2 mM) of TNB on cytotoxicity and cellular metabolic activity were examined using cultured astrocytes from Fischer-344 rats. The cytotoxicity was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Astrocyte metabolic activity was assessed by measuring the conversion of a tetrazolium salt to a formazan product. Additionally, the effects of oxygen tension on cellular metabolic activity were determined by varying oxygen tension via alteration of culture media depth. *In vitro*, the toxic concentration 50% (TC50) of TNB that induced cell death, was 16 μM following a 24 h exposure. However, the concentration of TNB that reduced cellular metabolic activity by 50%, was 29 μM following a 24 h exposure. In comparison with TNB, the concentration of *m*-dinitrobenzene (DNB) necessary to produce a 50% reduction in cellular metabolic activity was at least 50 fold greater than TNB. Varying the depth of the culture media did not influence the cellular metabolic activity in control or TNB treated astrocytes.

These results support the hypothesis that TNB induced neurotoxicosis could partially be mediated via injury to astrocytes, a major component of the blood brain barrier.

Introduction

1,3,5-Trinitrobenzene is an industrial chemical produced following the oxidation of trinitrotoluene (TNT) or the decarboxylation of trinitrobenzoic acid (Borges, 1991). TNB is a soil and water contaminant at installations that are currently or have been involved in the manufacture of explosives. Previous studies in Fischer-344 rats receiving single daily doses of trinitrobenzene over 6 to 10 days have resulted in methemoglobinemia, hemolytic anemia, testicular atrophy, and neurologic pathology (Chandra, 1989; Chandra et al., 1995; Chandra et al., 1995; Chandra et al., 1997; Chandra et al., 1997; Chandra et al., 1999). Ataxia progressing to recumbency is a manifestation of the neurologic abnormalities. In experimental intoxications with TNB, symmetrical morphological changes in the brain stem associated with the ataxia have been reported. These changes involve petechial hemorrhages and necrosis (malacia) with reactive gliosis in the cerebellar peduncles. The presence of vacuolation and associated extravasated serum proteins is an indication of vasogenic brain edema, which appears to be a critical event in TNB toxicity (Chandra et al., 1995; Chandra et al., 1999). The lesions appear primarily glial and vascular in origin, with secondary neuronal involvement.

The mechanisms by which TNB exerts its toxic action on the CNS are unclear, but previous investigators have determined that TNB mediated tissue damage is accompanied by breakdown of the blood brain barrier (BBB) with extravasation of albumin (Chandra et al., 1999). Astrocytes contribute to the maintenance and induction

of the BBB characteristics in the brain endothelium, including tightness of tight junctions (Stewart & Wiley, 1981; Butt et al., 1990; Rubin et al., 1991). Changes in the permeability of membranes are a common sequela of toxic insult at either the subcellular or cellular level.

The neurotoxic effects of dinitrobenzene (DNB) have been extensively studied both *in vitro* and *in vivo.* Dinitrobenzene has been shown to induce vacuolated lesions along with petechial hemorrhages and edematous swelling in the brain stem of rats (Philbert et al., 1987; Romero et al., 1991; Ray et al., 1994; Romero et al., 1995). Increases in blood flow and glucose consumption in areas of the brain susceptible to toxic insult have been shown to precede the onset of morphologic changes, suggesting an initial involvement of the vascular bed and subsequently the blood brain barrier in the development of the lesions (Romero et al., 1991; Romero et al., 1995).

Lactate dehydrogenase release *in vitro* has been used as an index of cytotoxicity in astrocytes (Romero et al., 1995; Romero et al., 1996). The basal metabolic rate has been suggested as being a possible critical factor in the neurotoxicosis of DNB. Cells with increased metabolic rates being more sensitive to damage. In single cultures, astrocytes have been shown to be more metabolically active than endothelial cells, which may contribute to the increased sensitivity of astrocytes to DNB. The highly toxic hydroxyl radical generated from H_2O_2 by the Fenton reaction has been theorized to play a partial role in DNB-induced toxicity in both astrocytes and endothelial cells. Excessive production of reactive oxygen species and other free radicals are also thought to significantly contribute to futile redox cycling in the toxicity of DNB (Romero et al., 1996).

Low oxygen tension favors the reduction of nitroaromatic and nitroheterocyclic compounds *in vitro* by nitroreductases, thus forming the nitro radical anion by a single

electron transfer from an electron donor, which is usually NADPH (Mason & Josephy, 1985; Biaglow et al., 1986). Higher oxygen tension favors the reaction of the nitro radical with molecular oxygen, which is usually faster than the ability of the enzyme to add a second electron, leading to production of superoxide radicals and the regeneration of the parent nitro compound (Mason, 1990). Previous studies have demonstrated that decreasing media depth in culture wells does not result in increased toxicity to type II pneumocytes from the superoxide generating compound paraquat (Hoet et al., 1997). However, others have observed increased toxicity to Hep G2 cells from paraquat by decreasing the culture well media depth (Qualls et al., 2000).

The major objectives of this study were to determine the levels of TNB cytotoxic to astrocytes in culture by measuring release of lactate dehydrogenase (LDH), and monitoring changes in cellular metabolic activity. Additional experiments were conducted to examine the influence of media depth changes on metabolic activity.

Materials and Methods

Materials

1,3,5-trinitrobenzene (99.83% purity) was obtained from Naval Surface Warfare Center and the purity of the compound was confirmed by High Performance Liquid Chromatography (HPLC). 1,3-Dinitrobenezene, Hank's Balanced Salt Solution, glucose, sucrose, sodium ascorbate and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal glial fibrillary acidic protein (GFAP) antibody was purchased from BioGenex (San Ramon, CA). CytoTox 96® Non-Radioactive Cytotoxicity Assay Kits and the CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kits were obtained from Promega Co. (Madison, WI). Dulbecco's Modified Eagle's Medium (DMEM), N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), and sodium pyruvate were purchased from JRH Biosciences (Lenexa, KS). Trypsin, Fetal Bovine Serum (FBS), penicillin G, streptomycin and L-glutamine were purchased from the Oklahoma Animal Disease Diagnostic Laboratory (Stillwater, OK). All tissue culture chemicals were of the highest analytical purity or endotoxin-tested grade.

Cell Culture

Primary cultures of type I astrocytes were prepared from the hippocampi of 2-day-old Fischer 344 rat pups using a modification of a previously established procedure (Booher & Sensenbrenner, 1972). The hippocampi were dissected in ice-cold Ca^{2+} -

and Mg^{2+} -free Hank's balanced salt solution (HBSS) containing 17 mM glucose, 22 mM sucrose, 10 mM HEPES, 100 U/ml penicillin G, and 100 mg/ml streptomycin at pH 7.3-7.4 and freed of meninges and choroid plexus. Tissue disaggregation was done by digestion in 0.125% trypsin at 37°C for 30 minutes. Digested tissue was filtered through sterile cheesecloth and trypsinization halted by addition of 10 ml FBS. The suspension was spun at 750 rpm for 10 minutes and then the supernate was removed. Cells were re-suspended in culture media and then seeded on poly-L-lysine-coated tissue culture dishes at a plating density of 25,000 cells/cm^2 and maintained in a water-saturated atmosphere of 5% CO_2 95% air. Culture medium consisted of DMEM containing FBS 10%, 1 mM sodium pyruvate, 0.057 mM sodium ascorbate, 2 mM glutamine, 26 mM sodium bicarbonate, 20 mM HEPES, 100 U/ml penicillin G and 100 mg/ml streptomycin. Culture medium was changed every 2 to 3 days until cells reached confluence (approximately 2 weeks). Cell contaminants were removed from type I astrocytes by shaking at 37°C for 24 hours with subsequent replacement of culture medium. Identification of at least 90% of the main cell population as astrocytes was based on positive staining with antibodies for GFAP (Raff et al., 1979).

Experiment One: Determination of Cytotoxicity

Single cultures of astrocytes were seeded on Costar® 96 well cell culture plates and allowed to reach confluence. Cultures were treated with different concentrations (0 μM, 0.002 μM, 0.02 μM, 0.2 μM, 2 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 1 mM, and 2 mM) of TNB in 100 μL of the medium (using 0.5% dimethyl sulfoxide, DMSO, as a vehicle) and allowed to incubate for 24 hours. Medium was not changed

during the experiments. Astrocyte cytotoxicity was assessed with the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit. Cell death was assessed by the percentage of lactate dehydrogenase (LDH) released into the culture medium upon cell lysis. Released LDH was measured with a 30-minute coupled enzymatic assay that converts NAD+ and lactate to pyruvate and NADH. 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was converted into a red formazan product in the presence of NADH that was detected colorimetrically at 490 nm with a 96 well plate reader. The amount of color formed was proportional to quantity of LDH released from lysed cells.

Experiment Two: Determination of Cellular Metabolic Activity

Astrocyte cultures that had reached confluence on Costar® 96 well culture plates were treated with different concentrations (0 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, 100 μM, and 125 μM) of TNB in 100 μL of the medium (using 0.5% DMSO, as a vehicle) and allowed to incubate for 24 hours. Medium was not changed during the experiments. Cellular metabolic activity was then compared in astrocytes exposed to varying concentrations of either TNB or *m*-DNB to assess differences in toxicity to these two nitroaromatic compounds. Metabolic activity of astrocytes was measured via [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] a water-soluble tetrazolium salt, in the presence of phenazine ethosulfate (PES), an intermediate electron acceptor. PES amplifies the MTS signal when it is converted to a formazan product that can be detected colorimetrically. The conversion of MTS to the formazan product is accomplished via NADPH or NADH by dehydrogenase enzymes in metabolically active cells. Cellular

metabolic activity was assessed in astrocytes with the cellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit. The reduction of MTS to the MTS formazan product was recorded at 490 nm with a 96 well plate reader. Reduction of the tetrazolium salt occurs in active mitochondria residing in living cells (Sun et al., 1997). This assay was also repeated on single cultures of astrocytes exposed to similar concentrations of DNB (0 mM, 0.5 mM, 1.0 mM, and 2.0 mM) used by Romero et al. (1996).

Experiment Three: Effects of Media Depth on Cellular Metabolic Activity

Costar® 96 well culture plates containing astrocyte monolayers were treated with different concentrations (0 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, and 90 μM) of TNB in the medium (using 0.5% DMSO, as a vehicle) and allowed to incubate for 2 hours. Different volumes (50, 100 and 200 μL) of media were used in each well to determine if increasing oxygen tension would increase cellular toxicity. Decreasing media volumes in individual culture chamber wells should allow for greater oxygen saturation of the media and oxygen availability for radical formation. Medium was not changed during the experiments. Cellular metabolic activity was assessed in astrocytes with the cellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit as described previously.

Data Analysis

All data were initially examined by analysis of variance (ANOVA) with a general linear model (GLM) using SAS (SAS Institute, Carey, NC). Mean ± standard deviation of astrocytic LDH release and MTS formazan formation for each group were calculated and compared to appropriate controls using Dunnett's test. Cytotoxicity, measured by LDH release from astrocytes, and metabolic activity, measured by MTS formazan formation, were both transformed using a probit by logarithmic dose scale. The toxic concentration 50% (TC50) and TNB quantity resulting in a 50% decrease in cellular metabolic activity were determined using linear regression of transformed data.

Results

Determination of Cytotoxicity

TNB at concentrations of 70 μM or higher produced a loss of continuity of the cell monolayer when cultured astrocytes were observed microscopically after 24 hours of exposure. Closely apposed contacts between cells were lost and the monolayer appeared to have gaps where cells had lysed in comparison to controls not exposed to TNB (Figures 1 & 2). Concentrations of 60 μM or less did not induce noticeable morphologic changes in astrocytes over 24 hours.

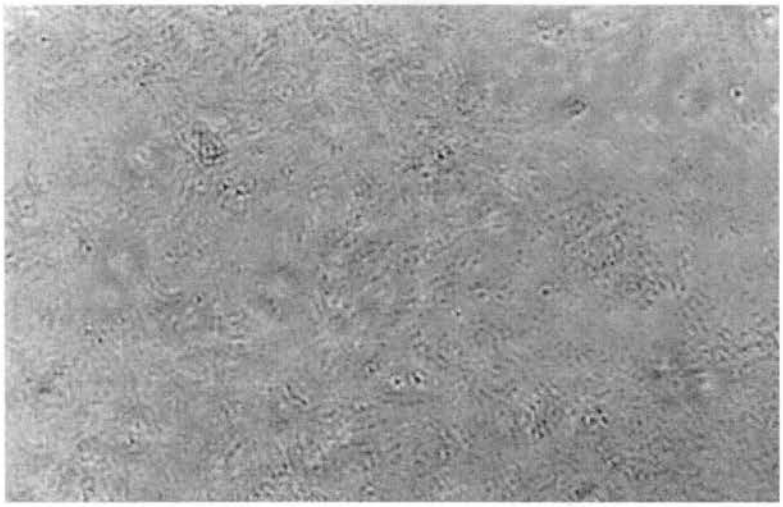

Figure 1. Morphologic appearance in phase-contrast light microscopy of control primary cultures of astrocytes. Cultures were treated with DMSO (0.5%) alone for 1 day. Control astrocytes display a confluent cell monolayer 7 days after seeding.

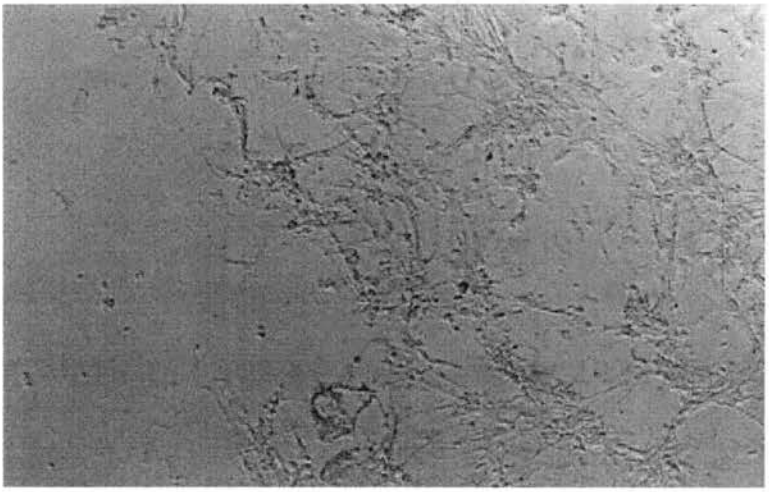

Figure 2. Morphologic appearance in phase-contrast light microscopy of TNB-treated primary cultures of astrocytes. Cultures were treated with 40 μM TNB dissolved in DMSO (0.5%) for 1 day. TNB-treated astrocytes display widespread cell death over the ensuing 24 hours.

The threshold TNB concentration that induced an increase in the percentage of dead astrocytes, measured by LDH release into the culture medium, was 20 μM after 24 hours exposure (Figure 3).

Transformation of the percentage cell death versus linear dose data to a probit response versus log dose produced a linear, dose-response curve. These lines allow for more precise predictions using linear regression analysis. The concentration of TNB eliciting 50% cell death in rat astrocytes after 24 hours incubation is 16 μM (Figure 4).

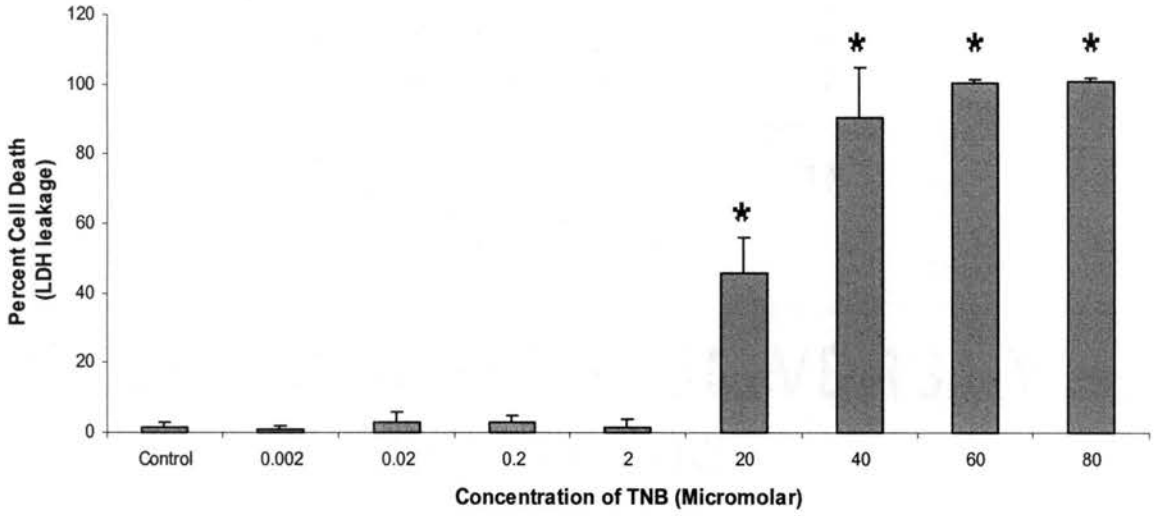

Figure 3. Dose dependent cell death measured by LDH release in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P <0.05).

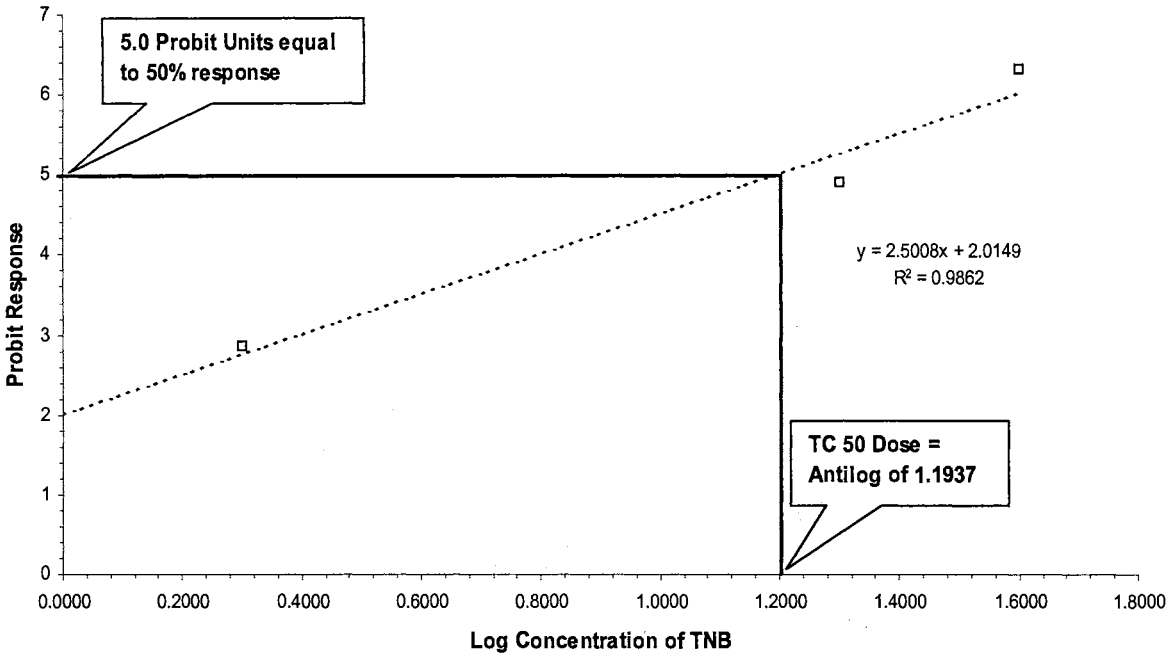

Figure 4. Toxic Concentration 50% (TC50) as determined by linear regression of a log-probit transformation of LDH release in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3,5-Trinitrobenzene.

Determination of Cellular Metabolic Activity

Cellular metabolic activity was assessed in cultured astrocytes after exposure to both TNB and DNB. Cellular metabolic activity was increased at 1.0 mM and significantly decreased in astrocytes exposed to 2.0 mM DNB (Figure 5).

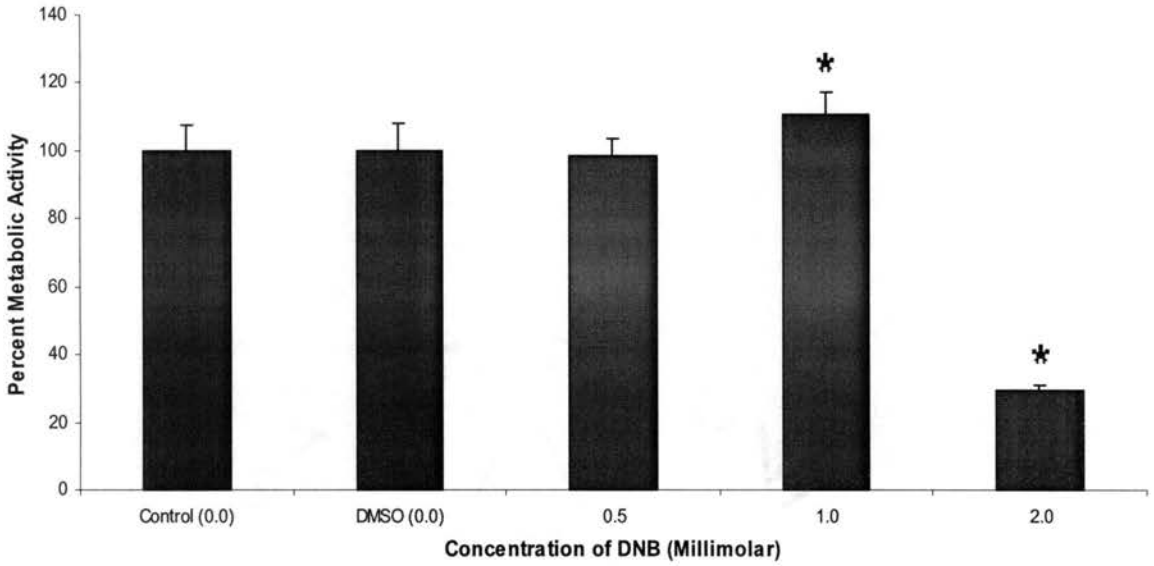

Figure 5. Assessment of metabolic activity measured by MTS reduction to a MTS formazan product in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3-Dinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P <0.05).

Cultured astrocytes displayed increased sensitivity to the toxic effects of TNB (micromolar range) in comparison with astrocytes treated with DNB (millimolar range) (Figure 6). Astrocytes displayed a 50% decrease in metabolic activity when exposed to 29 μM TNB (Figure 7).

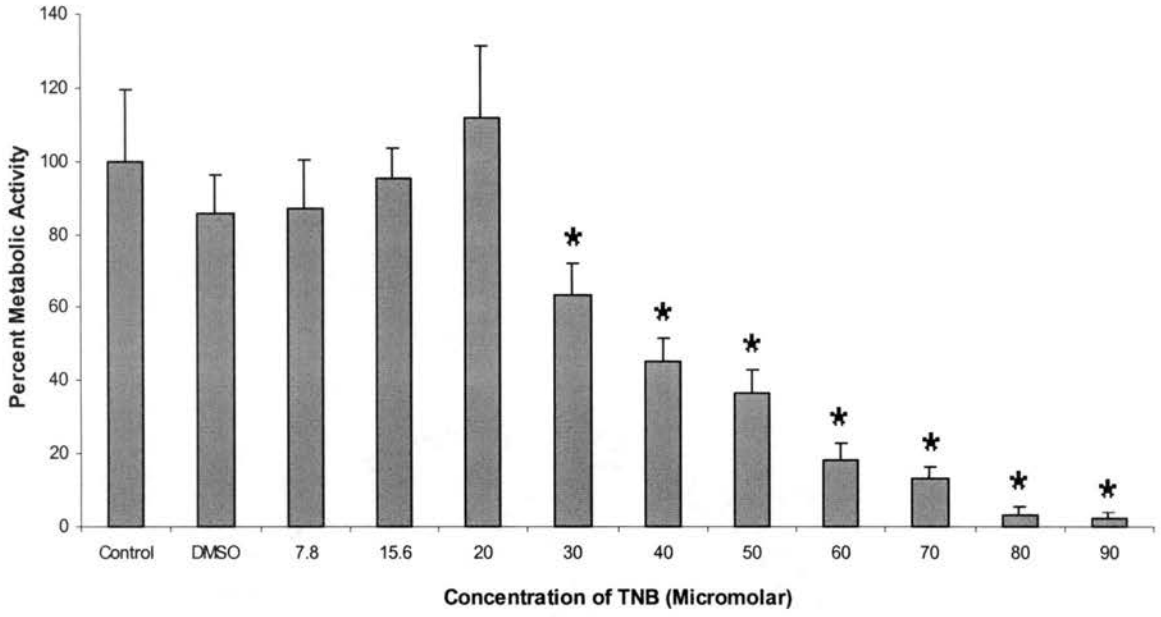

Figure 6. Percent metabolic activity measured by MTS reduction to a MTS formazan product in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P <0.05).

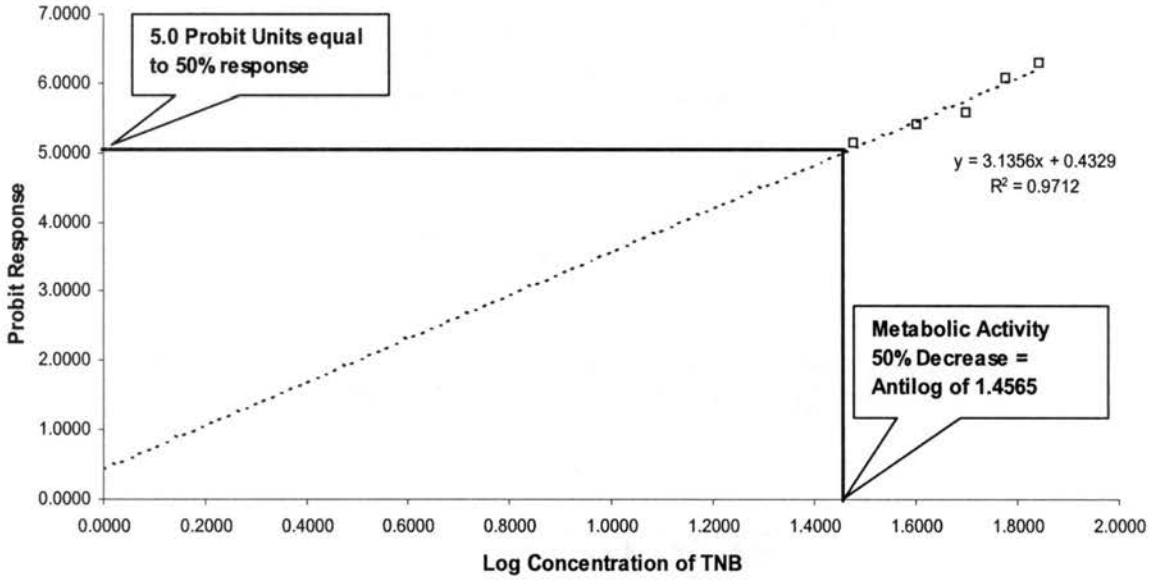

Figure 7. Metabolic activity decrease 50% as determined by linear regression of a log-probit transformation measured by MTS reduction to a MTS formazan product in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3,5-Trinitrobenzene.

Effects of Media Depth on Cellular Metabolic Activity

Changing media depth to allow greater oxygen penetration of the culture media layer did not significantly change cellular metabolic activity from the 3 volumes (50, 100 and 200 μL) used in this experiment (Figure 8). Different incubation times also did not change the results with different media depths (results not shown).

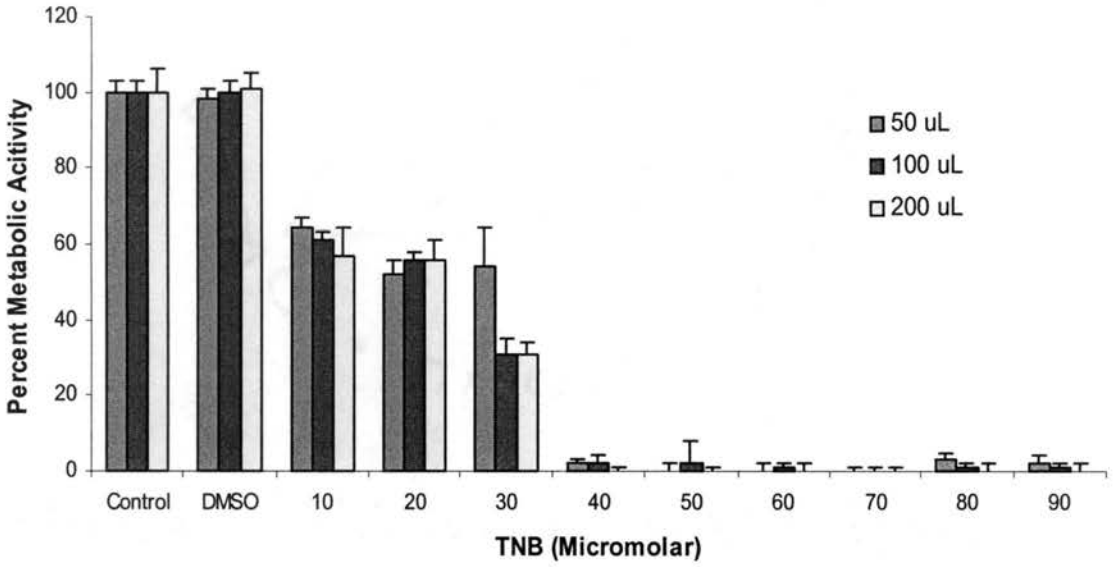

Figure 8. Metabolic activity measured by MTS reduction to a MTS formazan product in Fischer-344 rat astrocytes exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Different media volumes (50, 100 and 200 μL) were used to determine if increased oxygen tension would significantly decrease metabolic activity. Results are expressed as means ± SD.

Discussion

This study represents a new approach towards discovery of the neurotoxic mechanisms of TNB. All previous investigations with TNB neurotoxicosis have focused on *in vivo* exposure in the rat. Neuropathology induced by TNB has been shown to be accompanied by the breakdown of the blood-brain barrier (Chandra et al., 1999). *In vivo* work has helped to identify areas of brain pathology but has not examined the response of individual cells of these brain regions, specifically astrocytes and endothelial cells of the blood-brain barrier. This investigation was the first attempt to begin looking at individual cells, specifically astrocytes and their response to toxic concentrations of TNB. Investigations were also aimed at comparing the response of astrocytes exposed to TNB with DNB, another nitroaromatic compound producing lesions in similar regions of the rat brain.

The release of lactate dehydrogenase from single or mixed cultures of brain cells (astrocytes, endothelial cells, and neurons) is often used as a measure of cytotoxicity or loss of cellular viability (Romero et al., 1995; Romero et al., 1996; Sparapani et al., 1997; Deshpande & Nishino, 1998; Robb & Connor, 1998). The toxic concentration 50% (TC50) of TNB that induced cell death in cultured astrocytes was 16 μM following a 24 h exposure. Romero et al. (1996) reported dosage levels of 1.0 mM DNB resulting in approximately 10% loss of cell viability and concentrations of 2.0 mM causing greater than 90% cell death. The concentration of TNB required to elicit the same level of cytotoxicity was at least 50 times less than that of DNB. These results demonstrate that in single cultures of astrocytes, TNB has greater toxicity than DNB. Perhaps, the difference between TNB and DNB could be explained by potential differences in their metabolism or cell responses to their toxicity.

Cellular metabolic activity may play a critical role in the neurotoxicosis caused by TNB, cells with higher metabolic rates being more sensitive to damage. Investigators working with DNB have found a strong relationship between brain areas susceptible to damage in experimental energy deprivation syndromes and the regions displaying the highest metabolic rates (Ray et al., 1992; Cavanagh, 1993). Early increases in glucose consumption in these brain areas damaged by DNB have been shown to precede the morphologic lesion (Romero et al., 1995). The concentration of TNB that reduced cellular metabolic activity by 50%, was 29 μM following 24 h exposure. In comparison with TNB, the concentration of *m*-dinitrobenzene necessary to produce a 50% reduction in cellular metabolic activity was at least 50 fold greater than TNB. The dosage levels of TNB and DNB required to elicit a 50% change in metabolic activity were greater than the concentrations required for 50% cell death (as measured by LDH release). This might suggest that metabolic activity is a more accurate indicator of cell death than LDH release. To make sure that TNB alone was not reducing MTS to the MTS formazan product, culture wells were set up without astrocytes and media with TNB at different concentrations (similar to the metabolic activity experiment) was run via the MTS assay. No significant reduction of MTS occurred when TNB treated media was run via the MTS assay without astrocytes (data not shown). Astrocytes exhibited increases in metabolic activity at concentrations of 20 μM TNB and 1.0 mM DNB. These increases in metabolic activity were attributed to stimulation of these cells, with subsequent increased metabolic output in response to toxic insults (TNB and DNB).

Different volumes (50, 100 and 200 μL) of media were used in wells to determine if a mechanism requiring increased oxygen tension, such as redox cycling, would increase cellular toxicity. Decreasing media volumes in individual culture chamber wells

should allow for greater oxygen saturation of the media and oxygen availability for radical formation. Varying the depth of the culture media did not influence the cellular metabolic activity in control or TNB treated astrocytes.

Another striking finding of this study is the difference between toxicity observed between TNB and DNB *in vivo* and *in vitro*. Three 10 mg/kg doses of *m*-DNB are ultimately neurotoxic to rats, with lesions developing 12 hours after the third dose (Romero et al., 1991). Five to ten 71 mg/kg doses of TNB are neurotoxic to rats (either by observed clinical signs or histopathologic changes in the brain stem and cerebellum) (Chandra et al., 1995). Therefore, DNB appears to exhibit greater toxicity *in vivo* versus TNB. The concentration of TNB resulting in 50% cell death of cultured astrocytes (measured by LDH release) was 16 μM after 24 hours incubation. The concentration of DNB resulting in the same level of cell death of cultured astrocytes was between 1 and 2 mM after 24 hours incubation (Romero et al., 1996). Therefore, while DNB appears to exhibit greater toxicity *in vivo*, TNB displays increased toxicity *in vitro*. Several possible explanations for this include differences in solubility, different metabolites produced after metabolism in organs other than the brain, or differences in absorption in the gastrointestinal tract.

In summary, the results of these studies demonstrate the ability of astrocytes to be utilized in culture and quantify changes when exposed to TNB or other nitroaromatic compounds. This experimental model begins to examine astrocytes, a major component of the blood-brain barrier and their response to TNB. The determination of a range of *in vitro* TNB concentrations that can reliably reproduce astrocyte death (measured by LDH release or decreased metabolic activity) and should be a useful tool

for further cell culture investigations. Culture media depth does not appear to play a significant role in the pathogenesis of *in vitro* toxicity.

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CHAPTER V

AN *IN VITRO* STUDY OF 1,3,5-TRINITROBENZENE TOXICITY ON BOVINE AND HUMAN ENDOTHELIAL CELLS

Abstract

1,3,5-Trinitrobenzene (TNB) is an industrial chemical causing gliovascular lesions in the brain stem similar to those produced by thiamine deficiency and nitroaromatic compounds including *m*-dinitrobenzene. Endothelial cells, a major component of the blood brain barrier were used in culture to identify early preneuropathic indices of TNB-induced toxicity. The effects of varying concentrations (0 – 250 μM) of TNB on cytotoxicity and cellular metabolic activity were examined using cultured endothelial cells of bovine corneal and human umbilical vein origin. Cytotoxicity was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Endothelial cell metabolic activity was assessed by measuring the conversion of a tetrazolium salt to a formazan product. In bovine endothelial cells, the toxic concentration 50% (TC50) of TNB that induced cell death was 54 μM following 24 h exposure. Cellular metabolic activity was decreased by 50% in bovine and human endothelial cells exposed to concentrations of 49 and 45 μM TNB, respectively. These results suggest that when cultured alone, astrocytes (TC50 = 16 μM and 50% reduction in cellular metabolic activity = 29 μM) are more susceptible to cell death than endothelial cells when exposed to equivalent concentrations of TNB. Results of this study also confirm that this *in vitro* model could

be suitable for assessing the potential role of the blood brain barrier in TNB-induced encephalopathy.

Introduction

1,3,5-Trinitrobenzene is an industrial chemical produced following the oxidation of trinitrotoluene (TNT) or the decarboxylation of trinitrobenzoic acid (Borges, 1991). TNB is a soil and water contaminant at installations that are currently or have been involved in the manufacture of explosives. In Fischer-344 rats, trinitrobenzene exposure can result in methemoglobinemia, hemolytic anemia, testicular atrophy, and neurological abnormalities, such as ataxia progressing to recumbency when receiving multiple doses over 6 to 10 days. In experimental intoxications with TNB, symmetrical morphological changes in the brain stem associated with the ataxia have been reported. These changes involve petechial hemorrhages and necrosis (malacia) with reactive gliosis in the cerebellar peduncles. The presence of vacuolation and associated extravasated serum proteins is an indication of vasogenic brain edema, which appears to be a critical event in TNB toxicity (Chandra et al., 1995; Chandra et al., 1999). The lesions appear primarily glial and vascular in origin, with secondary neuronal involvement.

The mechanisms by which TNB exerts its toxic action on the CNS are unclear, but previous investigators have determined that TNB mediated tissue damage is accompanied by breakdown of the blood brain barrier (BBB) with extravasation of albumin (Chandra et al., 1999). The BBB of higher vertebrates is formed by the layer of endothelial cells lining the brain microvessels and the close association with the end feet of perivascular astrocytes (Abbott et al., 1992). Endothelial cells that comprise the BBB are different from peripheral endothelial cells in response to inductive factors found in

the nervous system allowing them to form highly resistant tight junctions and exhibit low rates of paracellular leakage and fluid-phase endocytosis (Rubin et al., 1991). Endothelial cells taken from non-neural areas of the body or even different animal species can take on characteristics of endothelial cells of the BBB when co-cultured with astrocytes (Stanness et al., 1996; Hayashi et al., 1997; Kuchler-Bopp et al., 1999). Brain endothelial cells deprived of astrocytic contact or conditioned media (increased cAMP levels) tend to lose many of their *in vivo* barrier properties such as the complexity of tight junctions, transendothelial electrical resistance and specific enzymatic activities (Tao-Cheng et al., 1987; Dehouck et al., 1990; Meyer et al., 1991; Rubin et al., 1991; Tontsch & Bauer, 1991; Raub et al., 1992).

Dinitrobenzene (DNB) has been extensively studied regarding its toxic effects on cultured endothelial cells. The basal metabolic rate has been suggested as being a possible critical factor in the neurotoxicosis of DNB. Cells with increased metabolic rates being more sensitive to damage. In single cultures, astrocytes have been shown to be more metabolically active than endothelial cells, which may contribute to the increased sensitivity observed to DNB by astrocytes. Also, differences in sensitivity to DNB have been observed between single and co-cultures of brain endothelial cells, but not of astrocytes, suggesting that astrocytes increase the susceptibility of endothelial cells to DNB toxicity. An enhanced generalized enzymatic activity as a result of cell differentiation in coculture may increase the production of reactive oxygen species in endothelial cells, and the depletion of reducing equivalents by futile redox cycling of the parent compound, with subsequent negative effects on the cell. In single cultures, differentiation of astrocytes by addition of cAMP analogs to the culture medium increases the susceptibility of astrocytes to DNB (Romero et al., 1996).

This study attempted to answer several questions. One aim was to determine levels of TNB cytotoxic to bovine endothelial cells by measuring release of lactate dehydrogenase (LDH) after 24 hours of incubation. Another objective was to determine cellular metabolic activity of bovine and human endothelial cells in culture after 24 hour TNB exposure. Cellular metabolic activity was then compared with astrocytes exposed to varying concentrations of TNB to gauge differences in toxicity to these different cell types.

Materials and Methods

Materials

1,3,5-trinitrobenzene (99.83% purity) was obtained from Naval Surface Warfare Center and the purity of the compound was confirmed by HPLC. Sodium bicarbonate, heparin, endothelial cell growth supplement (ECGF) and calf serum was purchased from Sigma Chemical Co. (St. Louis, MO). Human umbilical vein endothelial cells (CRL-1730), Ham's F12K medium, and Bovine corneal endothelial cells (CRL-2048) were obtained from American Type Culture Collection (Manassas, VA). CytoTox 96® Non-Radioactive Cytotoxicity Assay and the CellTiter 96® Aqueous One Solution Cell Proliferation Assay were obtained from Promega Co. (Madison, WI). Dulbecco's Modified Eagle's Medium (DMEM), HEPES, and sodium pyruvate were purchased from JRH Biosciences (Lenexa, KS). Trypsin, L-glutamine, and Fetal Bovine Serum (FBS) were purchased from the Oklahoma Animal Disease Diagnostic Laboratory (Stillwater,

OK). All tissue culture chemicals were of the highest analytical purity or endotoxin-tested grade.

Bovine Corneal Endothelial Cell Culture

Passaged cultures of Bovine corneal endothelial cells were obtained commercially and seeded on collagen coated plastic tissue culture dishes and maintained in a water-saturated atmosphere of 5% CO_2 95% air. Culture medium consisted of DMEM (85%) containing FBS (5%) and calf serum (10%). Culture medium was also supplemented with 2 mM glutamine and 26 mM sodium bicarbonate.

Culture medium was changed every 2 to 3 days until cells reached confluence (approximately 2 weeks).

Human Umbilical Vein Endothelial Cell Culture

Passaged cultures of Human umbilical vein endothelial cells were obtained commercially and seeded on collagen coated plastic tissue culture dishes and maintained in a water-saturated atmosphere of 5% CO_2 95% air. Culture medium consisted of Ham's F12K medium (with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate), 100 μg/ml heparin, 30 μg/ml endothelial cell growth supplement, and fetal bovine serum 10%.

Culture medium was changed every 2 to 3 days until cells reached confluence (approximately 2 weeks).

Experiment One: Determination of Bovine Endothelial Cell Cytotoxicity

Single cultures of bovine endothelial cells were seeded on Costar® 96 well cell culture plates and allowed to reach confluence. Cultures were treated with different concentrations (0 μM, 2 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM, 160 μM, 200 μM, and 250 μM) of TNB in 100 μL of the medium (using 0.5% dimethyl sulfoxide, DMSO, as a vehicle) and allowed to incubate for 24 hours. Medium was not changed during the experiments. Endothelial cell cytotoxicity was assessed with the CytoTox 96® Non-Radioactive Cytotoxicity Assay. Cell death was assessed by the percentage of lactate dehydrogenase (LDH) released into the culture medium upon cell lysis. Released LDH was measured with a 30-minute coupled enzymatic assay that converts NAD+ and lactate to pyruvate and NADH. 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was converted into a red formazan product in the presence of NADH that was detected colorimetrically at 490 nm with a 96 well plate reader. The amount of color formed was proportional to the quantity of LDH released from lysed cells.

Experiment Two: Determination of Cellular Metabolic Activity

Bovine and Human endothelial cells that had reached confluence on Costar® 96 well culture plates were treated with different concentrations (0 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, and 100 μM) of TNB in 100 μL of the medium (using 0.5% dimethyl sulfoxide, DMSO, as a vehicle) and allowed to incubate for 24 hours. Medium was not changed during the experiments. Metabolic activity of endothelial cells was measured via [3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] a water-soluble tetrazolium salt, in the presence of phenazine ethosulfate (PES), an intermediate electron acceptor. PES amplifies the MTS signal when it is converted to a formazan product that can be detected colorimetrically. The conversion of MTS to the formazan product is accomplished via NADPH or NADH by dehydrogenase enzymes in metabolically active cells. Cellular metabolic activity was assessed in Bovine and Human endothelial cells with the cellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit. The reduction of MTS to the MTS formazan product was recorded at 490 nm with a 96 well plate reader after 1.5 hours of incubation. Reduction of the tetrazolium salt occurs in active mitochondria residing in living cells (Sun et al., 1997).

Data Analysis

All data were initially examined by analysis of variance (ANOVA) with a general linear model (GLM) using SAS (SAS Institute, Carey, NC). Mean ± standard deviation of LDH release (Bovine endothelial cells) and MTS formazan formation (Bovine and Human endothelial cells) for each group were calculated and compared to appropriate controls using Dunnett's test. Cytotoxicity, measured by LDH release from Bovine endothelial cells, and metabolic activity, measured by MTS formazan formation, were transformed using a probit by logarithmic dose scale. The toxic concentration 50% (TC50) and TNB quantity resulting in a 50% decrease in cellular metabolic activity were determined using linear regression of transformed data.

Results

Cytotoxicity

Loss of continuity of the cell monolayer was observed microscopically in cultured bovine endothelial cells at concentrations of 90 μM TNB and higher after 24 hours exposure. Closely apposed contacts between cells were lost and the monolayer appeared to have gaps where cells had lysed. Concentrations of 80 μM or less did not induce noticeable morphologic changes in astrocytes over 24 hours. The lowest concentration (2 μM) of TNB used in the cytotoxicity experiment resulted in a 5.6% increase in cell death, measured by LDH release into the culture medium after 1 day of incubation (Figure 1). Greater than ninety-percent cell death was observed in bovine endothelial cells at dosage levels of 80 μM and higher.

Transformation of the percentage cell death versus linear dose data to a probit response versus log dose produced a linear, dose-response curve. These lines allow for more precise predictions using linear regression analysis. The concentration of TNB eliciting 50% cell death in bovine endothelial cells after 24 hours incubation is 54 μM (Figure 2).

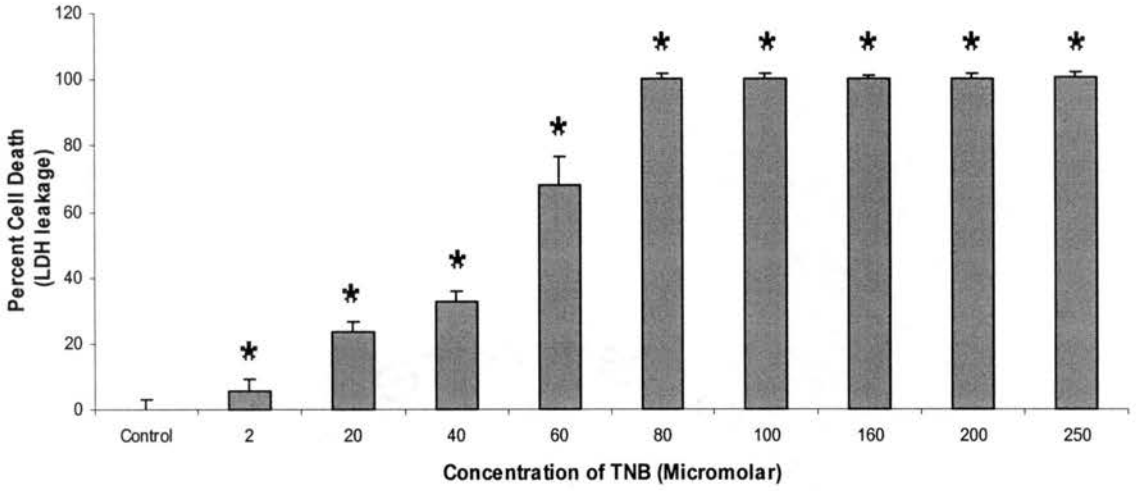

Figure 1. Dose dependent cell death measured by LDH release in bovine corneal endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P <0.05).

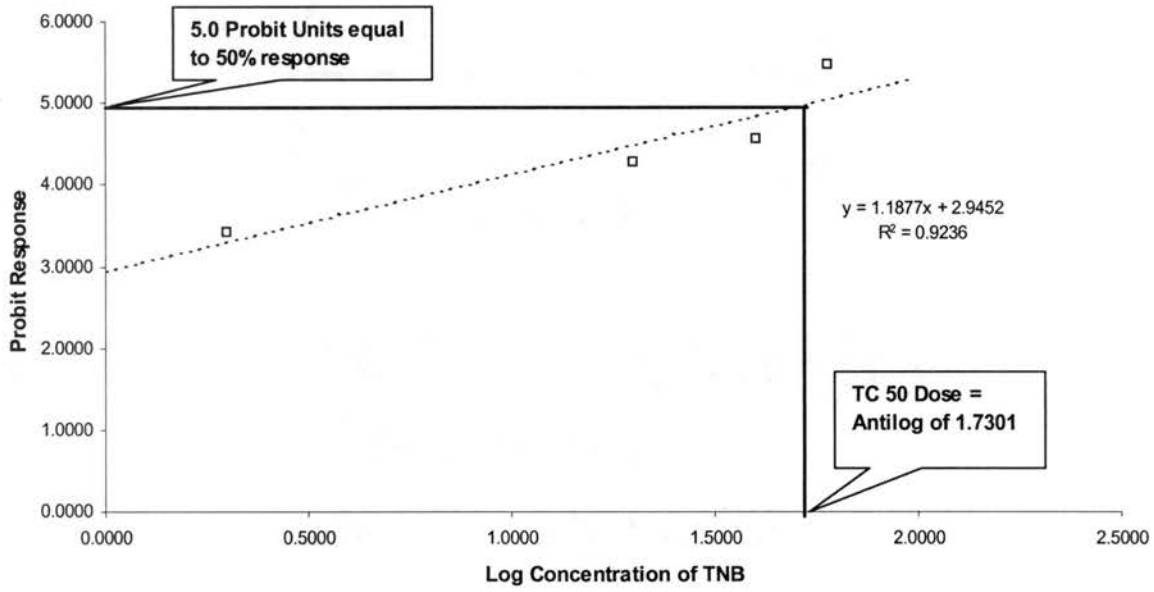

Figure 2. Toxic concentration 50% (TC50) as determined by linear regression of a log-probit transformation in bovine corneal endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene.

Cellular Metabolic Activity

Cellular metabolic activity was assessed in cultured Bovine and Human endothelial cells after exposure to TNB. Bovine endothelial cells did not significantly change metabolic activity in the vehicle control group containing DMSO at 0.5%. Human endothelial cells exhibited a 20% decrease in metabolic activity in the vehicle control group that was statistically significant. Cellular metabolic activity increased at 20 μM in both Bovine and Human endothelial cells from the vehicle control group. A significant decrease in metabolic activity was observed in Human cells at 30 μM and Bovine cells exposed to 40 μM TNB (Figures 3 and 4). Bovine endothelial cells displayed a 50% decrease in metabolic activity when exposed to 49 μM TNB (Figure 5). Human endothelial cells displayed a 50% decrease in metabolic activity when exposed to 45 M TNB (Figure 6).

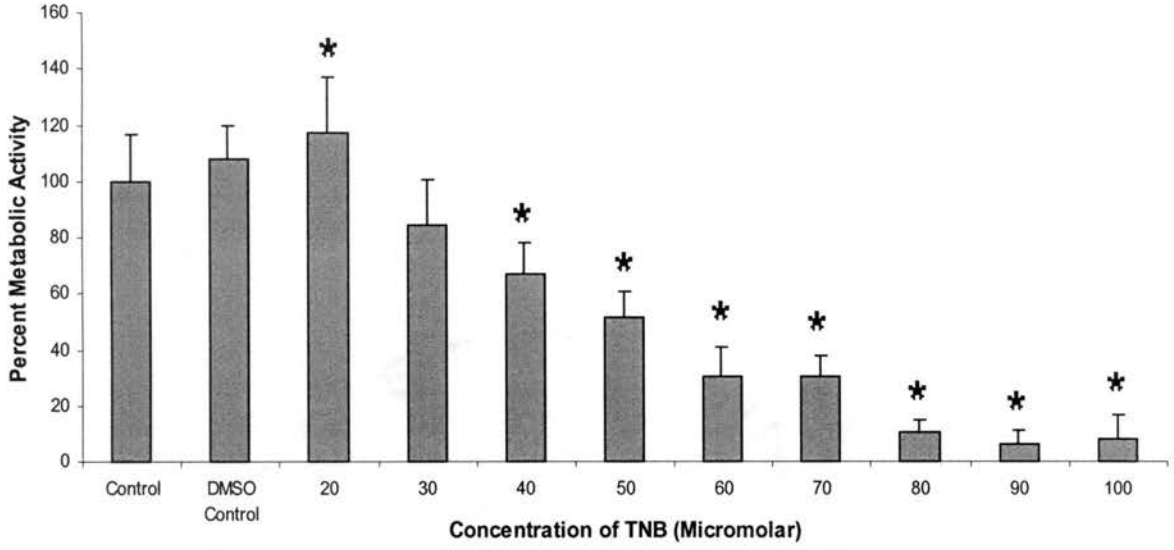

Figure 3. Percent metabolic activity measured by MTS reduction to a MTS formazan product in bovine corneal endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P < 0.5).

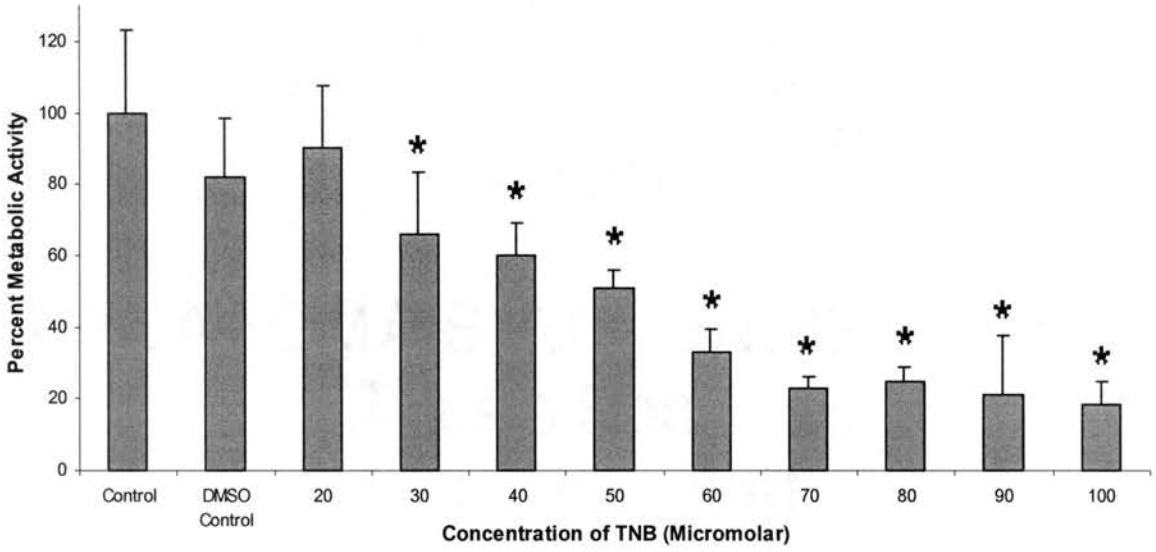

Figure 4. Percent metabolic activity measured by MTS reduction to a MTS formazan product in human umbilical vein endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene. Results are expressed as means ± SD (*=Statistically Significant, P < 0.5).

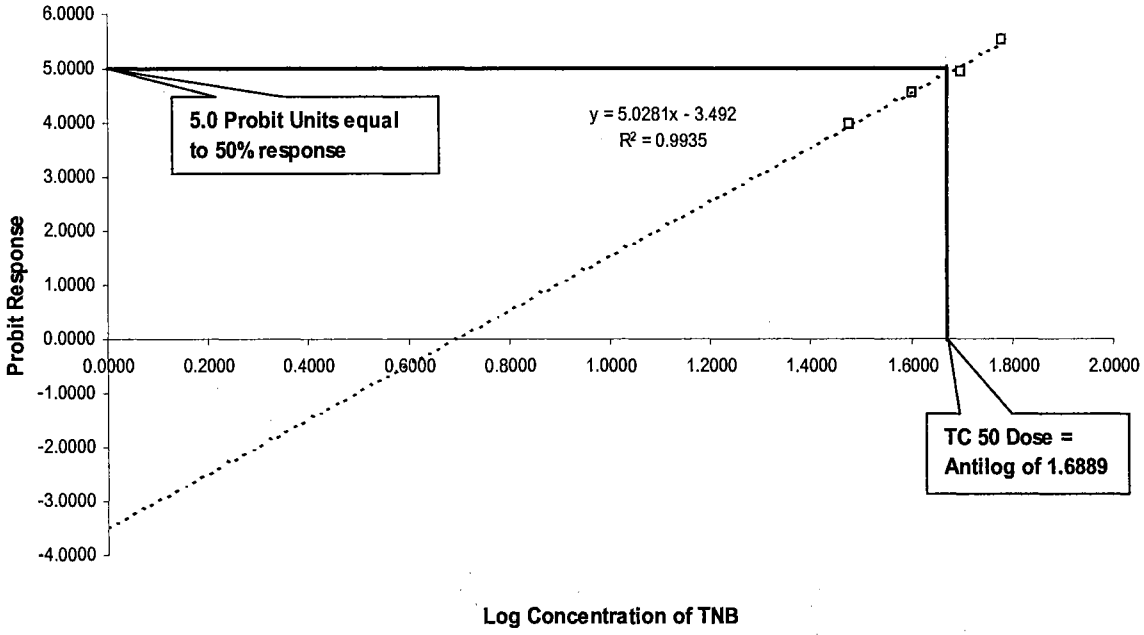

Figure 5. Metabolic activity decrease 50% as determined by linear regression of a log-probit transformation measured by MTS reduction to a MTS formazan product in bovine corneal endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene.

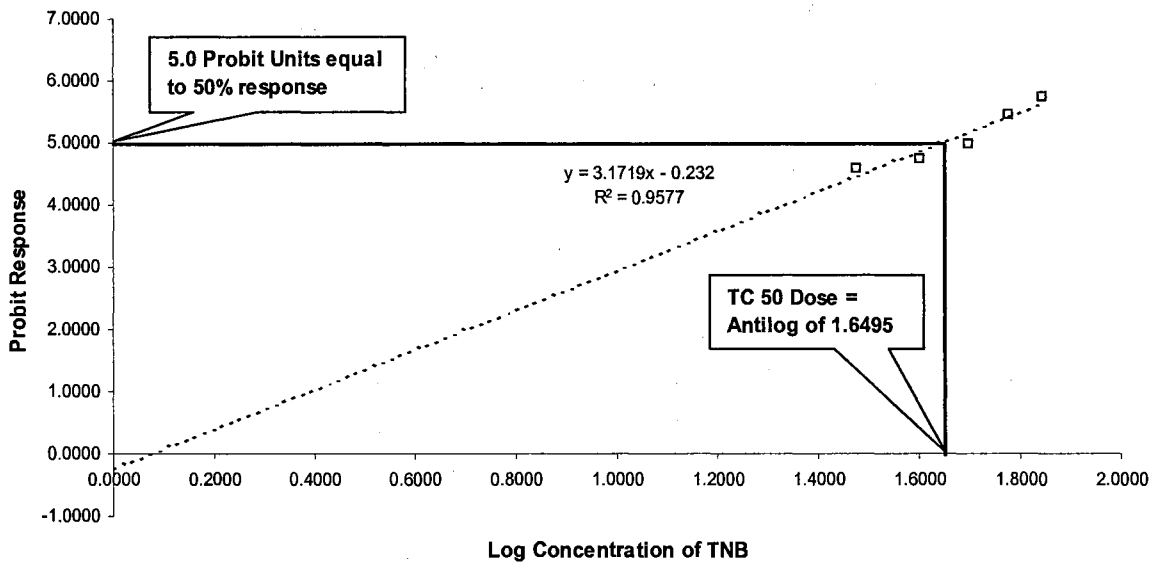

Figure 6. Metabolic activity decrease 50% as determined by linear regression of a log-probit transformation measured by MTS reduction to a MTS formazan product in human umbilical vein endothelial cells exposed to increasing concentrations of 1,3,5-Trinitrobenzene.

Discussion

This study using endothelial cells in culture represents the first attempt towards elucidating a partial mechanism for the encephalopathy induced by TNB. All previous investigations with TNB neurotoxicosis have focused on *in vivo* exposure in the rat. Neuropathology induced by TNB has been shown to be accompanied by the breakdown of the blood-brain barrier (Chandra et al., 1999). *In vivo* work has helped to identify areas of brain pathology but has not examined the response of individual cells of these brain regions, specifically astrocytes and endothelial cells of the blood-brain barrier.

The release of lactate dehydrogenase from single or mixed cultures of brain cells (astrocytes, endothelial cells, and neurons) is often used as a measure of cytotoxicity or loss of cellular viability (Romero et al., 1995; Romero et al., 1996; Sparapani et al., 1997; Deshpande & Nishino, 1998; Robb & Connor, 1998). The toxic concentration 50% (TC50) of TNB that induced cell death in cultured bovine corneal endothelial cells was 54 μM following a 24 h exposure. Unfortunately, we were unable to successfully obtain reliable results with LDH release from human umbilical vein endothelial cells. Romero et al. (1996) reported dosage levels of 2.0 mM causing approximately 30% cell death. The concentration of TNB required to elicit the same level of cytotoxicity was at least 50 times less than that of DNB. These results demonstrate that in single cultures of endothelial cells, TNB has greater toxicity than DNB. Perhaps, the difference between TNB and DNB could be explained by potential differences in their metabolism or cell responses to their toxicity.

Cellular metabolic activity may play a critical role in the neurotoxicosis caused by TNB, cells with higher metabolic rates being more sensitive to damage. Previous studies with DNB have shown a strong relationship between brain areas susceptible to

damage in experimental energy deprivation syndromes and the regions displaying the highest metabolic rates (Ray et al., 1992; Cavanagh, 1993). Early increases in glucose consumption in these brain areas damaged by DNB have been shown to precede the morphologic lesion (Romero et al., 1995). The concentration of TNB that reduced cellular metabolic activity in bovine corneal endothelial cells by 50%, was 49 μM following 24 h exposure. In comparison with bovine corneal endothelial cells, the concentration of TNB necessary to produce a 50% reduction in cellular metabolic activity in human umbilical vein endothelial cells was 45 μM following 24 h exposure. Thus, it is interesting to observe that endothelial cells of bovine and human origin behave in a similar manner in respect to TNB induced alteration of metabolic activity. The dosage levels of TNB required to elicit a 50% change in metabolic activity were less than the concentrations required for 50% cell death (as measured by LDH release). Bovine corneal and human umbilical vein endothelial cells exhibited increases in metabolic activity at concentrations of 20 μM TNB. These increases in metabolic activity were attributed to stimulation of these cells, with subsequent increased metabolic output in response to toxic insults (TNB and DNB). These results suggest that when cultured alone, astrocytes (TC50 = 16 μM and 50% reduction in cellular metabolic activity = 29 μM) are more susceptible to cell death than endothelial cells when exposed to equivalent concentrations of TNB.

Endothelial cells taken from non-neural areas of the body or even different animal species can take on characteristics of endothelial cells of the BBB when co-cultured with astrocytes (Stanness et al., 1996; Hayashi et al., 1997; Kuchler-Bopp et al., 1999). Brain endothelial cells deprived of astrocytic contact or conditioned media (increased cAMP levels) tend to lose many of their *in vivo* barrier properties such as the complexity

of tight junctions, transendothelial electrical resistance and specific enzymatic activities (Tao-Cheng et al., 1987; Dehouck et al., 1990; Meyer et al., 1991; Rubin et al., 1991; Tontsch & Bauer, 1991; Raub et al., 1992).

Bovine corneal endothelial cells produce an extracellular matrix that can be used to support the growth of rat neuronal cells and astrocytes. Future investigations need to utilize co-culture of endothelial cells with astrocytes to gain a better appreciation of the consequences of TNB exposure to the blood brain barrier.

In summary, the results of these studies demonstrate for the first time that endothelial cells, a major component of the blood-brain barrier, can be used to assess the toxic effects of TNB. These results, along with data from experimentation with astrocytes in culture indicate that TNB-induced encephalopathy could be partially mediated by its toxic effects either on endothelial cells or astrocytes. Future studies using these two cell types in co-culture should provide a more definitive indication of the direct effects of TNB on the blood brain barrier. Of course, one should attempt to use both astrocytes and endothelial cells of rodent origin. In addition, the determination of a range of *in vitro* TNB concentrations that can reliably reproduce endothelial and astrocyte cell death (measured by LDH release or decreased metabolic activity) should be a useful tool for further co-culture investigations.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

The results of all of the previous studies indicate that 1,3,5-trinitrobenzene induces gliovascular lesions in the brain stem and cerebellum of Fischer-344 rats after once daily gavage at 71 mg/kg for 10 days. In the experiments designed to modulate endogenous nitric oxide and thiamine levels (Chapter II), the brains of all rats receiving TNB only had histological lesions consisting of gliosis and malacia with accompanying hemorrhage in the cerebellar peduncles and brain stem. Lesions were observed in the superior olivary nuclei, inferior colliculi, superior cerebellar peduncle, interpositus nuclei and lateral cerebellar nuclei of the medulla and cerebellum. Pretreatment with L-arginine did not alter the lesions induced by TNB except for no apparent pathology in the inferior colliculi. Similarly pretreatment with L-NAME did not induce changes in lesions in comparison with TNB administered alone. Rats treated with pyrithiamine and TNB had lesions consistent with rats dosed with TNB only. Thiamine supplementation along with TNB gavage did not prevent lesions similar in appearance to those induced by TNB only.

Oral administration of TNB induced an increase in whole blood thiamine levels compared to controls. However, co-administration of thiamine along with TNB produced a four-fold increase in whole blood thiamine levels compared with a two-fold increase when thiamine was administered alone. While a trend towards a decrease in whole blood thiamine levels was observed in rats dosed with pyrithiamine and TNB, and rats receiving pyrithiamine only, the decrease was not significant when compared with control animals.

Assessing the role of oxidative stress in the induced encephalopathy (Chapter III) pretreatment with vitamin E (*dl* α-tocopheryl acetate) did not induce changes in lesions in comparison with TNB administered alone. Rats treated with vitamin C and TNB had lesions consistent with rats dosed with TNB only. N-acetylcysteine (NAC) supplementation along with TNB gavage did not prevent lesions similar in appearance to those induced by TNB only. Similarly, combination of NAC with either vitamin E or vitamin C did not induce changes in lesions when compared with TNB administered alone.

The *in vitro* studies with astrocytes (Chapter IV) assessed cytotoxicity by lactate dehydrogenase (LDH) leakage into the culture medium. Astrocyte metabolic activity was quantified by measuring the conversion of a tetrazolium salt to a formazan product. Additionally, the effects of oxidative stress on cellular metabolic activity were determined by varying oxygen tension via alteration of culture media depth. *In vitro*, the toxic concentration 50% (TC50) of TNB that induced cell death, was 16 μM following a 24 h exposure. However, the concentration of TNB that reduced cellular metabolic activity by 50%, was 29 μM following a 24 h exposure. In comparison with TNB, the concentration of *m*-dinitrobenzene (DNB) necessary to produce a 50% reduction in cellular metabolic activity was at least 50 fold greater than TNB. Varying the depth of the culture media did not influence the cellular metabolic activity in control or TNB treated astrocytes.

The *in vitro* studies with using endothelial cells (Chapter V), assessed cytotoxicity by lactate dehydrogenase (LDH) leakage into the culture medium. Endothelial cell metabolic activity was assessed by measuring the conversion of a tetrazolium salt to a formazan product. In bovine endothelial cells, the toxic concentration 50% (TC50) of TNB that induced cell death was 54 μM following 24 h exposure. Cellular metabolic

activity was decreased by 50% in bovine and human endothelial cells exposed to concentrations of 49 and 45 μM TNB, respectively. These results suggest that when cultured alone, astrocytes (TC50 = 16 μM and 50% reduction in cellular metabolic activity = 29 μM) are more susceptible to cell death than endothelial cells when exposed to equivalent concentrations of TNB.

In summary, it is concluded that both morphologic and histopathologic changes in rats receiving TNB only were consistent with those described by Chandra et al. (1995). The results presented in the study that attempts to modulate endogenous NO and thiamine levels and protect against oxidative stress do not lend strong support to the hypothesis that nitric oxide, thiamine or oxidative stress play a significant role in TNB-induced neurotoxicosis. Interestingly, results presented in this study indicate that oral administration of TNB alone induced an increase in whole blood thiamine levels. The dose of pyrithiamine, a known thiamine antagonist, used in this study did not alter the normal morphologic and histologic features of the brain stem and cerebellum. This can be explained by the fact that adequate induction of thiamine deficiency in rodents dosed with pyrithiamine requires placing them on a thiamine deficient diet. However, the data derived from experiments using either astrocytes or endothelial cells in culture partially support that TNB-induced neurotoxicosis might be mediated by damage to the blood brain barrier.

VITA

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