Cytotoxicity and Expression of c-fos, HSP70, and GADD45/153 Proteins in Human Liver Carcinoma (HepG2) Cells Exposed to Dinitrotoluenes

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Abstract: Dinitrotoluenes (DNTs) are byproducts of the explosive trinitrotoluene (TNT), and exist as a mixture of 2 to 6 isomers, with 2,4-DNT and 2,6-DNT being the most significant. The main route of human exposure at ammunition facilities is inhalation. The primary targets of DNTs toxicity are the hematopoietic system, cardiovascular system, nervous system and reproductive system. In factory workers, exposure to DNTs has been linked to many adverse health effects, including: cyanosis, vertigo, headache, metallic taste, dyspnea, weakness and lassitude, loss of appetite, nausea, and vomiting. Other symptoms including pain or paraesthesia in extremities, abdominal discomfort, tremors, paralysis, chest pain, and unconsciousness have been documented. An association between DNTs exposure and increased risk of hepatocellular carcinomas and subcutaneous tumors in rats, as well as renal tumors in mice, has been established. This research was therefore designed targeting the liver to assess the cellular and molecular responses of human liver carcinoma cells following exposure to 2,4-DNT and 2,6-DNT. Cytotoxicity was evaluated using the MTT assay. Upon 48 hrs of exposure, LC₅₀ values of 245 ± 14.72 µg/mL, and 300 ± 5.92 µg/mL were recorded for 2,6-DNT and 2,4-DNT respectively, indicating that both DNTs are moderately toxic, and 2,6-DNT is slightly more toxic to HepG2 cells than 2,4-DNT. A dose response relationship was recorded with respect to the cytotoxicity of both DNTs. Western blot analysis resulted in a significant expression (p<0.05) of the 70-kDa heat shock protein in 2,6-DNT-treated cells compared to the control cells and at the 200 µg/mL dose for 2,4-DNT. A statistically significant expression in c-fos was also observed at the 200 and 250 µg/mL treatment level for 2,4- and 2,6-DNT, respectively. However, no statistically significant expression of this protooncogene-related protein was observed at the doses of 0, 100, or 300 µg/mL or within the dose range of 0-200 µg/mL for 2,6-DNT. The 45-kDa growth arrest and damage protein was significantly expressed at the dose range of 200 – 250µg/mL for 2,6-DNT and at the dose range of 200 - 400µg/mL for 2,4-DNT. Expression of 153-kDa growth arrest and DNA damage protein was significant at the 100, 200, and 250µg/mL doses for 2,6-DNT and at the 200 µg/mL dose for 2,4-DNT. Overall, these results indicate the potential of DNTs to induce cytotoxic, proteotoxic (HSP70), and genotoxic (GADD45/153) effects, as well as oxidative stress and pro-inflammatory reactions (c-fos).

Keywords: dinitrotoluenes, cytotoxicity, c-fos, HSP70, GADD45, GADD153, HepG2 cells.

Introduction

Dinitrotoluenes (DNTs) [CH₃C₆H₃(NO₂)₂] are nitroaromatic compounds that are produced industrially and released into the environment as a result of human activities. DNTs appear as pale yellow crystalline solids and exist as a mixture of 2 to 6 isomers, among which 2,4-DNT and 2,6-DNT are the most significant at room temperature [1]. The compounds 2,4-dinitrotoluene (2,4-DNT; 1-methyl-1,3-dinitrobenzene; CAS Reg. No. 606-20-2) and 2,6-dinitrotoluene (2,6-DNT; 1-methyl-1,3-dinitrobenzene; CAS Reg. No. 606-20-2) have a molecular weight of 182.14, melting points of 71 °C (2,4-DNT) and 66°C (2,6-DNT) and boiling points of 300 °C (2,4-DNT) and 260°C (2,6-DNT) [2]. They are soluble in alcohol, ether, acetone, and benzene, but only slightly soluble in water – 270 parts per million (ppm) at 22°C (2,4-DNT) and 180 ppm at 20°C (2,6-DNT) [2-5].

Although both 2,4-DNT and 2,6-DNT are transformation products of 2,4,6-trinitrotoluene [4], the main pathway of their production is through the dinitration of toluene with nitric acid in the presence of...
sulfuric acid. This reaction produces a commercial grade mixture, also called technical grade (tg), containing about 75% 2,4-DNT isomer, 20% 2,6-DNT isomer, and 5% of the other DNT isomers [6-8]. Dinitrotoluenes are used in the manufacture of dyes, in munitions as smokeless propellant powder, and as gelatinizing and plasticizing agents in both commercial and military explosive compositions [6]. Their major use is in the synthesis of toluenediamine, the organic intermediate used in the production of polyurethane [7]. In the United States, about 500 persons are estimated to be potentially exposed yearly to 2,4-DNT and 2,6-DNT during the production of munitions and explosives.

Absorption of 2,4-DNT and 2,6-DNT occurs through the gastrointestinal tract, the respiratory tract, and the skin in most species [9]. From a study of 33 workers in a tg-DNT explosives factory, Woolen et al. [10] reported that the skin was probably the primary route of exposure in these workers, and the lungs constituted the secondary route because of low atmospheric levels of DNTs. Humans absorb both isomers of DNT following dermal or inhalation exposure. Urine is the primary route of elimination of both DNT isomers in most animals including rodents, rabbits, dogs, and monkeys; in CD-1 and B6C3F1 mice and in Wistar rats, but the main route of elimination is the feces. The percent absorption of the DNT isomers is difficult to assess because biliary excretion is significant in most animals; males excrete more of the 2,4-DNT isomer and its metabolites in the bile and less in urine than do females [7].

No systematic studies have been done on the distribution of DNTs in tissues of potentially exposed individuals. Trace amounts of 2,4-DNT and 2,6-DNT have been reported in blood samples of workers employed at a tg-DNT explosives factory [10]. Radiotracer studies with animals have shown slow amounts of radioactivity were found in the liver, blood, kidneys, brain, and skeletal muscle of mice, rats, rabbits, dogs, and monkeys following oral exposure to radio labeled 2,4-DNT [6, 11]. Peak levels of radioactivity were observed in the blood of male rats 6 hours after a single oral dose of [3H] 2,4-DNT [12].

Although the main route of human exposure at ammunition facilities is inhalation, dermal contact and inadvertent ingestion may also be substantial. Such exposures have been linked to many health effects in factory workers including cyanosis, vertigo, headache, metallic taste, dyspnea, weakness, and lassitude, loss of appetite, nausea and vomiting [6, 13, 14]. Other symptoms including pain or paralysis in extremities, abdominal discomfort, tremors, paralysis, chest pain and unconsciousness have been reported [13, 9].

The International Agency for Research on Cancer (IARC) and the U.S. EPA have classified DNT as a probable human carcinogen (Group B2). Epidemiologic data point to the possible urothelial carcinogenicity of DNT in highly and long term exposed miners. As a systemic toxicant, DNT has a potential of causing adverse health effects that target the hematopoietic system (pallor, cyanosis, anemia, and leukocytosis), the cardiovascular system (ischemic heart disease), the nervous system (muscular weakness, headache, dizziness, nausea, insomnia, and tingling pains in the extremities), and the reproductive system (reduction of sperm counts, alteration of sperm morphology and aspermato genesis). The incidence of subcutaneous tumors and hepatocellular carcinomas in rats increased after exposure to 2,4-DNT and 2,6-DNT[14].

Epidemiologic studies of DNT toxicity have been limited to small groups of workers who were occupationally exposed at various ammunition production facilities. It has therefore been difficult to clearly define the health effects of DNTs with a high degree of confidence because of the multigenic nature of occupational exposure.

Methemoglobinemia has been reported as the first clinical sign of DNT toxicity, followed by cyanosis when the methemoglobin concentration rises above 15 mg/L [13, 10]. Gastrointestinal effects of DNT exposure are characterized by nausea and vomiting, secondary to methemoglobinemia. In humans, long-term exposure can cause jaundice. Incidents of poisoning resulting from inhalation of 2,4-DNT are primarily described in the early literature; with symptoms including marked cyanosis, headache, palpitations, tightness in the chest, insomnia, lack of appetite, impaired reflexes, nystagmus, and tremors following exposure to 2,4-DNT [15].

Cellular response to systemic toxicants or other deleterious forms of stress is characterized by the activation of stress-inducible transcription factors that up-regulate target genes. The regulation of stress gene expression is intimately linked to signaling pathways that coordinate long-term cell adaptation or programmed cell death. Moreover, cytotoxic stresses can influence the synthesis of protective proteins that are associated with biotransformation activity, cell cycle progression, DNA-impairment, proliferative machinery, oxidative damage, and protein perturbations. Recent evidence in our laboratory has shown that DNT transcriptionally activates a novel set of stress genes in human liver carcinoma cells. In the present study, we hypothesize that DNT treatment of HepG2 cells will induce cytotoxicity and increase the level of stress proteins that may play a role in the molecular events leading to toxicity and tumorigenesis.

**Materials and Methods**

**Chemicals**

2,4-Dinitrotoluene (CH$_2$C$_6$H$_3$(NO$_2$)$_2$), CAS Reg. No. 121-14-2, Lot No. 306-22A and 2,6-Dinitrotoluene (CH$_2$C$_6$H$_3$(NO$_2$)$_2$), CAS Reg. No. 606-20-2, Lot No. 295-21A were purchased from Chem Service (West Chester, PA). All culture media and medium supplements, pre-cast SDS-PAGE gels, c-fos, HSP70, GADD45 and GADD153 primary and secondary antibodies, and BCP/NBT color development substrate were purchased from commercially available sources.

**Culture of HepG2 Cells**

Human liver carcinoma, HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA). In the laboratory, they were stored in liquid nitrogen until use. They were next thawed by gentle agitation of their containers (vials) for 2 minutes
in a water bath at 37°C. After thawing, the content of each vial was transferred to a 75cm² tissue culture flask, diluted with DMEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, and incubated at 37°C in a 5% CO₂ incubator to allow the cells to grow, and form a monolayer in the flask. Subsequently, cells grown to 80-95% confluence were washed with phosphate buffer saline (PBS), trypsinized with 10mL of 0.25% (w/v) trypsin-0.03% (w/v) EDTA, diluted, counted (1 x 10⁶), and seeded in three 25 mm² flasks prior to chemical treatment.

**Cytotoxicity Experiment**

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed for assessing cytotoxicity in HepG₂ cells exposed to serial doses (0–300µg/mL) of 2,4-DNT and (0–250 µg/mL) of 2,6-DNT. This experiment was conducted following the experimental protocol that is routinely used in our laboratory [16]. HepG₂ cells are dosed with various concentrations of 2,4- and 2,6-DNT in 1% dimethyl sulfoxide DMSO and incubated at 37°C for 48 hrs, and the MTT assay for cell viability is performed using a microplate reader at 550nm to determine the cell viability of HepG₂ to 2,4- and 2,6-DNT and the lethal concentration (LC₅₀).

**Western Blot Analysis**

The Western Blot analysis was conducted to determine specific cellular response proteins (c-fos, GADD45, GADD153, HSP70) at 48 hrs of DNT exposure. About 1 x 10⁶ HepG₂ cells were grown to 80-95% confluency, and treated with 0, 100, 200, and 300 µg 2,4-DNT/mL and 0, 50, 100, 150, 200, and 250 µg 2,6-DNT/mL for 48 hrs. Control flasks were also made without DNT treatment. Five hundred µL of sample buffer (0.2 mol/L Tris, pH 6.8, 1% SDS, 30% glycerol, 7.5% β-mercaptoethanol, 0.1% bromophenol blue) was added to each flask and cells were mechanically dislodged and collected in microcentrifuge tubes. Whole cell extracts (20µg) from HepG₂ cells containing an equal volume of sample buffer were heated at 100°C for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel. Samples were then transferred onto a nitrocellulose membrane on ice and the membrane blocked (Tris buffered saline with 5% nonfat dry milk, 0.1 Tween 20) for 1 hr at room temperature. Detection of membrane-bound proteins induced by DNT was carried out using specific primary antibodies that recognize the proteins of interest. Subsequently, the reaction was reprobed with an alkaline conjugated anti-mouse IgG antibody for c-fos, GADD45, GADD153 and HSP70. BCIP/NBT color substrate was incorporated to develop protein bands. Immunoblot 1-D protein bands were assessed for relative abundance by Total Lab computer software (Nonlinear USA Inc. Durham, NC).

**Results**

**Cytotoxicity Assay**

The cytotoxic effect of 2,4-DNT on HepG₂ cells is shown in Figure 1. A classical dose-response pattern was demonstrated with respect to the cytotoxicity of 2,4-dinitrotoluene. The percentages of cell viability were 100.0 ± 0%, 88.18 ± 3.31%, 74.51 ± 4.31%, 50.15 ± 5.31%, 22.00 ± 6.53%, 26.07 ± 7.08%, 23.40 ± 7.76, 20.00 ± 10.99, and 21.54 ± 8.14 for 0, 100, 200, 300, 400, 500, 600, 700, and 800 µg/mL of 2,4-dinitrotoluene, respectively. Upon 48 hours of exposure the dose (LD₅₀) required to produce 50% reduction in the viability of HepG₂ cells was computed to be 300 ± 5.92 µg 2,4-DNT/mL. This result indicates that 2,4-DNT is moderately toxic to HepG₂ cells.

**Figure 1:** Cytotoxicity of 2,4-dinitrotoluene to HepG₂ cells

The MTT result of the cytotoxic effect of 2,6-dinitrotoluene on human liver carcinoma cells (HepG₂) following 48 hours of exposure is shown in Figure 2. This figure also indicates a strong dose-response relationship with respect to the cytotoxicity of 2,6-dinitrotoluene to HepG₂ cells. The percentages of cell viability were 100.0 ± 0%, 109.25 ± 15.42%, 105.73 ± 25.69%, 86.60 ± 16.89 %, 59.43 ± 19.10%, 45.45 ± 31.93%, 21.75 ± 37.22, 14.74 ± 23.62, and 8.94 ± 8.46 for 0, 50, 100, 150, 200, 250, 300, 350, and 400 µg/mL of 2,6-dinitrotoluene, respectively. There was a gradual decrease in cell viability with increasing doses of 2,6-dinitrotoluene. Upon 48 hours of exposure the dose (LD₅₀) required to produce 50% reduction in the viability of HepG₂ cells was computed to be 245.0 ± 14.72µg/mL. This result indicates that 2,6-DNT is moderately toxic to HepG₂ cells.

**Figure 2:** Cytotoxicity of 2,6-dinitrotoluene to HepG₂ cells.
Western and Densitometric Analyses

c-fos expressions in 2,4- and 2,6-DNT-treated HepG2 cells are shown in figures 3 & 4. c-fos was significantly expressed in the western blot and densitometric analysis of 2,4- and 2,6-dinitrotoluene at the 200µg/mL and 250µg/mL treatment levels, respectively. However, no statistically significant expression of this protooncogene-related protein was observed at the doses of 0, 100, or 300µg/mL for 2,4-dinitrotoluene or within the dose ranges of 0-200µg/mL for 2,6-dinitrotoluene.

![Figure 3: Western Blot and densitometric analysis of c-fos expression in 2,4-DNT treated HepG2 cells.](image1)

![Figure 4: Western Blot and densitometric analysis of c-fos expression in 2,6-DNT treated HepG2 cells.](image2)

Growth arrest and DNA damage (GADD) expressions in HepG2 cells treated with 2,4- and 2,6-DNT are shown in figures 5 & 6. The 45-kDa GADD protein was significantly over expressed at the 100 - 400 µg/mL dose range for 2,4-dinitrotoluene and at the dose levels of 200 and 250 µg/mL for 2,6-dinitrotoluene.

Expression of GADD153 protein was significant at the 200µg/mL dose for 2,4-dinitrotoluene and at the 100, 200, and 250 µg/mL doses for 2,6-dinitrotoluene. The western blot and densitometric analysis are shown in figures 7 & 8.

HSP70 expressions for 2,4-and 2,6-dinitrotoluene are shown in figures 9 & 10 respectively. There was a significant expression of the 70-kDa heat shock protein from western blot analysis (p<0.05) at the 200 µg/mL dose for 2,4-dinitrotoluene and a significant expression of the 70-kDa heat shock protein (p<0.05) of 2,6-dinitrotoluene compared to the control cells.

![Figure 5: Western Blot and densitometric analysis of GADD45 expression in 2,4-DNT treated HepG2 cells.](image3)

![Figure 6: Western Blot and densitometric analysis of GADD45 expression in 2,6-DNT treated HepG2 cells.](image4)

![Figure 7: Western Blot and densitometric analysis of GADD153 expression in 2,4-DNT treated HepG2 cells.](image5)

![Figure 8: Western blot and densitometric analysis of GADD153 expression in 2,6-DNT treated HepG2 cells.](image6)
Hepatotoxic effects include liver discoloration, and reticulocytosis, and an increase in Heinz bodies. Findings are methemoglobinemia, anemia, nervous system, and reproductive system are the targets for 2,6-DNT. The primary targets of DNT toxicity are (reduction of sperm counts, alteration of sperm morphology, and aspermatogenesis) [6, 13, 7, 9].

Experimental animals indicate that the blood, liver, morphology, and aspermatogenesis) [6, 13, 7, 9].

Densitometric analyses of HSP70 expression in 2,4-DNT treated HepG2 cells.

Discussion

Cytotoxicity

Cytotoxicity has been defined as the cell killing property of a chemical compound independent from the mechanism of death. Cytotoxicity assays measure the negative effects of the isomers, 2,4- and 2,6-dinitrotoluene, on cells. In the present study, it was demonstrated that different doses of 2,4- and 2,6-DNT induce cytotoxicity in human liver carcinoma cells. Data obtained from this research clearly demonstrate that under similar exposure conditions, the cytotoxicity of the dinitrotoluenes is dose-dependent with LD50 values of 300 ± 5.92µg/mL for 2,4-DNT and 245 ± 14.72µg/mL for 2,6-DNT. The primary targets of DNT toxicity are the hematopoietic system (pallor, cyanosis, anemia, and leukocytosis), the cardiovascular system (ischemic heart disease), the nervous system muscular weakness, headache, dizziness, nausea, insomnia, and tingling pains in the extremities), and the reproductive system (reduction in sperm counts, alteration of sperm morphology, and aspermatogenesis) [6, 13, 7, 9]. Subchronic and chronic oral toxicity studies with experimental animals indicate that the blood, liver, nervous system, and reproductive system are the targets affected by 2,4-DNT. The most common hematologic findings are methemoglobinemia, anemia, reticulocytosis, and an increase in Heinz bodies. Hepatotoxic effects include liver discoloration, and proliferative alterations of hepatocytes and bile duct epithelium. Neuromuscular effects, ranging from tremors and ataxia to convulsions, are more severe in dogs than in rodents. Reproductive effects are characterized by alterations of spermatogenesis, testicular atrophy, and ovarian dysfunction [17, 14, 18, 11, 19, 20].

Recent studies on the cytotoxicity of selected munitions compounds reported that 2,4,6-Trinitrotoluene has a relatively higher toxicity to human liver carcinoma cells (showing a LC50 of 105 ± 6 µg/mL) compared to its two metabolites, 2,4- and 2,6-DNT with LC50, greater than 300 µg/mL at 48 hours of exposure [34]. Other organic compounds such as pentachlorophenol (PCP), a biocidal chemical used in agriculture and as a wood preservative, have shown similar but greater cytotoxic effects to HepG2 cells than 2,4- and 2,6-DNT. PCP has a LD50 of 23.0 ± 5.6 µg/mL compared to 300 ± 5.92 µg/mL and 245 ± 14.72 µg/mL for 2,4- and 2,6-DNT respectively. Based on this information, it can be stated that 2,4- and 2,6-DNT are less toxic than PCP and 2,4,6-Trinitrotoluene. The order of toxicity is PCP>TNT>2,6-DNT>2,4-DNT.

An association between DNT exposure and increased risk of hepatobiliary cancer has been reported from a retrospective mortality study involving 4,989 workers exposed to DNT (isomer composition not specified) and 7,436 unexposed controls at an U.S. Army Munitions facility [19]. Exposure to 2,4-DNT containing trace amounts of 2,6-DNT was found to induce an increased incidence of hepatocellular carcinomas and subcutaneous tumors in rats and of renal tumors in male mice [14]. Exposure to tg-DNT induced hepatocellular carcinomas in rats [20, 21]. The results of isomer-specific DNT carcinogenicity studies have suggested, however, that 2,6-DNT rather than 2,4-DNT is the primary hepatocarcinogen in tg-DNT [20-23].

It has been reported that 2,6-DNT must undergo reduction in the gastrointestinal tract to become genotoxic [24, 25]. The compound is absorbed in the small intestine and metabolized in the liver to 2,6-dinitrobenzyl alcohol, which is then conjugated with glucuronic acid before excretion in the bile. The gastrointestinal flora then hydrolyzes the glucuronic linkage and reduces one or both of the nitro groups. The amino derivative is then reabsorbed and oxidized in the liver where it binds to DNA [4].

Western blot and Densitometric Analysis

Substantial expressions of GADD45 were observed for 2,4- and 2,6-DNT. Densitometric analysis revealed significant expressions at the 100 - 400 µg/mL dose range and the dose range of 200 – 250 µg/mL for 2,4- and 2,6-DNT, respectively. Substantial expressions of GADD153 were observed for 2,4- and 2,6-DNT. Densitometric analysis revealed significant expressions at the 200µg/mL dose and the doses of 100, 200 and 250 µg/mL for 2,4- and 2,6-DNT, respectively. These proteins are associated with cell cycle regulation and genotoxicity. The expression of these proteins indicates a potential damage at the genomic level. This damage may be associated with alterations in DNA sequence, as well as conformational changes in its helical structure [26].
The c-fos has been indicated as a mammalian UV response pathway, which appears to have an important role in the response to DNA damaging agents such as methyl methane sulfonate and 4-nitroquinoline N-oxide [27]. From our studies, we found strong expressions at the doses of 200 µg/mL and 250 µg/mL for 2,4- and 2,6-DNT, respectively. This protein is an integral part of the AP-1 transcriptional complex along with other nuclear proto-oncogenes including c-jun [28, 29]. The c-fos protein responds to DNA damage and oxidative stress [30]. Increased heat shock protein expression may be a result of alterations in protein structure through adducts formation [31]. DNT-induced expression of heat shock is an indication of its proteotoxic effects in HepG2 cells [16, 32, 33]. The significant expression of the 70-kDa heat shock protein (HSP70) was shown at the 200 µg/mL [16, 32, 33]. The significant expression of the 70-kDa protein responds to protein damage and oxidative stress [30].

Conclusions

Data obtained from this research indicate that 2,4- and 2,6-dinitrotoluene are moderately cytotoxic to transformed human liver cells, with 48 hrs-LD₅₀ values of 300 ± 5.92 µg/mL and 245 ± 14.72 µg/mL, respectively. Western Blot and densitometric analyses resulted in a strong dose-response relationship with respect to GADD153 and HSP70 expression in HepG2 cells for 2,6-dinitrotoluene. At the molecular level, the proteotoxic and genotoxic effects, and oxidative stress and pro-inflammatory reactions of 2,4- and 2,6-DNT were characterized by the over expression HSP70, GADD45/153, and c-fos proteins.

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