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Brain oxidative stress after dermal and subcutaneous exposure of T-2 toxin in mice

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ABSTRACT

T-2 toxin belongs to group of mycotoxins and is found as a natural contaminant in cereals, feed and vegetables. In the present study we evaluated acute toxicity of dermal and subcutaneous exposure of T-2 toxin on brain oxidative stress in mice. Mice were exposed to 1 LD50 of T-2 toxin either by dermal (5.94 mg/kg) or subcutaneous (1.54 mg/kg body weight) route and sacrificed at 1, 3 and 7 days post-exposure. T-2 toxin treated animals showed time dependent increase in reactive oxygen species generation, glutathione depletion, lipid peroxidation and protein carbonyl content in brain in both the routes of exposure. Gene expression profile of antioxidant enzymes showed significant increase in superoxide dismutase and catalase in percutaenous route and glutathione reducatse and glutathione peroxidase in subcutaneous route. Immunoblot analysis of antioxidant enzymes correlated with gene expression profile. T-2 toxin exposure resulted in down regulation of transcription factor Nrf2 and its downstream target genes of phase II detoxifying enzymes NQO1, Gclc, Gclm and hemeoxygenase-1. Results of our study show that percutaneously and subcutaneously applied T-2 toxin can cause brain oxidative damage possibly after crossing blood–brain barrier by altering its permeability.

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1. Introduction

T-2 toxin which belongs to the type A group is a non-macrocyclic trichothecene. It is mainly produced by Fusarium species, and is the most potent naturally occurring trichothecene. Potentially hazardous concentrations of the T-2 toxin can occur in agricultural products (WHO (World Health Organization), 1990). At high doses, T-2 toxin can cause shock-like syndrome that can result in death. It is a major causative agent in fatal alimentary toxic aleukia (ATA) in humans affecting mucosa and immune system. Severe poisoning results in prostration, weakness, ataxia, collapse, reduced cardiac output, shock and death (Sudakin, 2003). The toxicity of T-2 toxin by the inhalation route of exposure (LCt50 range: 200–5800 mg. min/m^3) is similar to that observed for mustards or Lewisite (Creasia et al., 1987). However, the lethality of T-2 toxin by dermal route is higher than that for liquid mustard. T-2 toxin is about 400 fold more potent than sulfur mustard in producing skin injury (Bunner et al., 1985). Even very low concentration of T-2 toxin poses a significant hazard to human GI immune function (Li et al., 2006).

A variety of mechanisms of action is proposed for T-2 toxin. In addition to its known mechanism of action of protein synthesis inhibition, T-2 toxin can impair the production of antibodies (Li et al., 2006), alter membrane functions, reduce lymphocyte proliferation (Kamalavenkatesh et al., 2005) and alter the maturation process of dendritic cells (Hymery et al., 2006). There is a limited evidence for tumorigenicity of T-2 toxin in experimental animals. It induced hepatocellular and pulmonary adenomas in male mice. Several studies in mice and rats indicate that toxin cause toxicity and proliferative changes in the oesophagus and fore stomach epithelium (Scientific Committee on Food, 2001). In vitro, T-2 toxin causes apoptosis in various cell types like HL60, Jurkat, U937, Vero cells and human hepatoma cells (Bouaziz et al., 2008). The involvement of oxidative stress and activation of various signaling pathways like MAP kinases, caspases have been shown in T-2 toxin induced apoptosis in vitro (Bouaziz et al., 2008; Chaudhari et al., 2009b).

The effects of T-2 toxin on central nervous system have received limited attention. The acute toxicity of T-2 toxin and other trichothecene mycotoxins varies with animal species and routes of administration of toxins (Fairhurst et al., 1987). Acute T-2 toxin toxicosis is characterized by hemorrhages, sepsis and cardiopulmonary failure (Kravchenko et al., 1996). Most of the published reports in various animal models are on T-2 toxin exposure by oral, intraperitoneal or intratracheal route. There is no report on the comparative toxicity profile after dermal (percutaneous) and subcutaneous route of T-2 toxin exposure in any animal model and data on brain oxidative stress.

Previous in vivo studies with T-2 toxin have demonstrated the ability of trichothecenes to induce neurotoxic events when toxin was injected into brain tissue directly. Studies have shown that ingestion of T-2 toxin leads to changes in amino acid permeability across blood–brain barrier (BBB), which could lead to neurological

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effects observed in animals exposed to trichothecenes (Wang et al., 1998). Low levels of T-2 toxin were responsible for the changes in the metabolism of brain biogenic monoamines (Boyd et al., 1988).

The objective of the present study was to evaluate the comparative acute toxicity of percutaneous (dermal) 5 and subcutaneous exposure of T-2 toxin on brain oxidative damage in mice. Mechanism of brain oxidative damage and recovery profile was assessed in a time course study (1, 3, 7 days post-exposure) in terms of (a) gene expression of primary antioxidant enzymes and phase II antioxidant enzymes, (b) protein expression and (c) oxidative protein damage. The results of our study clearly show significant T-2 toxin induced time and route dependent effects on oxidative damage and persistence of some of the effects even after 7 days of post-exposure.

2. Materials and methods

2.1. Chemicals

T-2 toxin was obtained from Alexis Biochemicals (Switzerland). Reduced glutathione (GSH) was from Across (Belgium). O-phthaldialdehyde (OPT) was from Fluka (USA). Antibodies against antioxidant enzymes Gpx, GR, GS, GST, CAT and SOD were obtained from Santa Cruz Biotechnology, Inc. (Europe). Anti-mouse HRP conjugated antibody was obtained from DAKO (Denmark). RNeasy protect mini kit and real-time PCR primers for antioxidant genes such as GPx, GST, GR, GS, CAT, SOD, Nrf-2, Hmox-1, Gclc, GClm and NQO1 were obtained from Qiagen, GmbH. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA) unless otherwise mentioned.

2.2. Animals and toxin exposure

Swiss albino female mice weighing between 22 and 24 g body mass from establishment's animal facility were used for the study. The animals were housed in polypropylene cages with dust-free rice husk as bedding material, and were provided with pellet food (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. The care and maintenance of the animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. This study has the approval of Institute's ethical committee on animal experimentation.

T-2 toxin was dissolved in minimum amount of DMSO and diluted in PBS before use. The mice were housed four to a cage and allowed to acclimatize 7 days prior to dosing. A day before percutaneous exposure of T-2 toxin, hair on the back of the animals was closely clipped using a pair of scissors. For percutaneous route (*p.c.*), the diluted toxin solution was smeared uniformly on the back of the animals on a circular area of 1.5 cm diameter, using a gas tight syringe (Harvard Apparatus, USA). Subcutaneous injection was given using the gas tight syringe on the back of the animals. Three day LD50 of T-2 toxin by *p.c.* and *s.c.* route of exposure was determined by Gad and Weil's method (Gad and Weil, 1989). For time course study, animals were divided into four groups of six animals each for each time point (0, 1, 3 and 7 days) of *p.c.* and *s.c.* route. The mice were kept in separate groups as 0 (control), 1, 3 and 7 days post-exposure. Mice were administered 1 LD50 of T-2 toxin by either *p.c.* (5.94 mg/kg body mass) or *s.c.* (1.57 mg/kg body mass) route. Control mice received equal volume DMSO by respective routes.

2.3. Estimation of ROS and GSH

Estimation of ROS in brain homogenate was carried out according to the protocol of Socci et al., (1999). Brain homogenate 0.25% was made in 40 mM Tris HCl pH 7.4 and was kept on ice, sample were divided into two equal halves. In one part 40 μl of 1.25 mM DCFDA in methanol was added and in other fraction only 40 μl of methanol was added and was used as control for autofluorescence. Incubation was done in ice for 15 min and fluorescence was observed at wavelength of 480 nm excitation and 525 nm emission. Unit for ROS were expressed in nmol/min/mg of protein.

2.4. Lipid peroxidation

Brain lipid peroxidation was measured by determining malondialdehyde (MDA) following the procedure of Ohkawa et al. (1979). A 10% brain homogenate was prepared in 1.15% KCl. Samples containing 100 μl of brain homogenate were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 ml with distilled water and heated to 95 °C for 120 min. After cooling and centrifugation at 15,000 rpm for 10 min, the supernatant fraction was isolated and the absorbance measured at 532 nm.

2.5. Determination of oxidative protein damage

Carbonyl levels in the brain were used as indicators of oxidative protein damage. Protein carbonyl content was quantitated by the protein carbonyl assay (Levine et al., 1990; Lushchak et al., 2005). The brain homogenate (10% w/v) was made in phosphate buffered saline (PBS, pH 7.4) containing protease inhibitor cocktail. The homogenate was centrifuged at 10,000*g* at 4 °C for 15 min and the protein carbonyls in the supernatants were analyzed by the method described. Briefly, 100 μl of mouse brain homogenate was incubated with 400 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) or 400 μl of 2.5 M HCl. After 1 h incubation at room temperature in dark, 0.5 ml of 20% trichloroacetic acid (TCA) was added, and then washed with 0.5 ml 10% TCA, followed by 0.5 ml mixture of ethanol/ethyl acetate (1:1). The protein pellets were dissolved in 250 μl of 6 M guanidine hydrochloride and centrifuged at 10,000*g* for 10 min at 4 °C. The OD of the supernatants was read at 370 nm. Protein carbonyls were calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.6. Real-time RT-PCR studies

Thirty milligrams of brain tissue was used to extract the total RNA from treated and control animals. RNA from liver tissue was isolated using RNeasy protect mini kit (Qiagen, GmbH) following manufacturer's protocol. The integrity and concentration of RNA was determined by Bioanalyzer 2100 (Agilent Technologies, USA). RNA is stored at −80 °C for further use. Real-time RT-PCR was carried out for the selected genes using gene specific primers from Qiagen and Qiagen SYBR® Green one-step qRT-PCR kit. The Stratagene Mx3005p system was used to monitor the SYBR® Green signal at the end of each extension period for 40 cycles. 100ng RNA was used for each gene The thermal profile consist of 10 min of reverse transcription at 50 °C one cycle and 5 min of polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting temperature (Tm) with the melting curve analysis software of the Mx3005p. The threshold cycle (Ct) of gene of interest and housekeeping gene (HK), and the difference between their Ct values (△Ct) were determined. Relative changes of gene expression were calculated by the following formula as described by Livak and Schmittgen (2001) and the data is represented as fold up/down regulation.

$$\text{Fold Change} = 2^{-\Delta\Delta Ct}$$

where △△ Ct = (Ct of gene of interest, treated−Ct of HK gene, treated)−(Ct of gene of interest, control−Ct of HK gene, control)Ct = threshold cycle number, HK = house keeping gene

We have considered only those genes, which are two fold or more (up or down regulated), than control as significant.

2.7. Western blot analysis

Brain tissue samples were processed for SDS–PAGE followed by Western blot for the analysis of expression of GST, GPX, GR, GS, CAT and SOD. The brain tissues from control and various treatment samples were homogenized with 5–10 volumes of lysis buffer (10 mM HEPES pH 7.4, 42 mM KCl, 50 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM DTT, 2 mM PMSF, 1X complete protease inhibitor cocktail). Cellular debris were spun down at 8000*g* for 20 min and supernatants were used as whole protein extract. The total protein concentration was estimated using Bio-Rad method. Fifty micrograms of protein from each sample was separated on SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane filter, using an electro-blotting apparatus. Membranes were incubated in blocking solution containing 5% non-fat dried milk in PBST buffer (PBS buffer containing 0.1% Tween-20) for 1 h at room temperature, followed by over night incubation at 4 °C in platform shaker with various primary antibodies at specified dilutions: Polyclonal antibodies against GPx, GS, GR, CAT, SOD (1:700); anti-GST (1:800). Monoclonal anti-β-actin (1:20000) which detects β-actin (42 kDa) was used as protein loading control. All antibodies are diluted in PBST with 5% milk powder. The membranes were washed four times in PBST for 15 min, followed by incubation for 2 h in horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit secondary antibody used at 1:80,000 dilutions. The membranes were washed again and developed using an enhanced chemiluminescent detection system (ProteoQwest™ Chemiluminescent Western blotting kit, Sigma) according to manufacturer's protocol and the image was taken on Pierce CL-XPosure™ X-ray films. The results of Western blots were quantified with image analyzer (Bio-Rad Quantity One software).

2.8. Statistical analysis

Data were analyzed by one-way ANOVA followed Dunnet's test for comparison between control and treatment groups. Data were expressed as mean ± SE of four animals per treatment. The level of significance was set at $p \leqslant 0.05$. All experiments were repeated at least twice.

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