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Tributyltin induces oxidative damage, inflammation and apoptosis via disturbance in blood–brain barrier and metal homeostasis in cerebral cortex of rat brain: An *in vivo* and *in vitro* study

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ABSTRACT

Tributyltin (TBT), a member of the organotin family, is primarily used for its biocidal activity. Persistent environmental levels of TBT pose threat to the ecosystem. Since neurotoxic influence of TBT remains elusive, we therefore, studied its effect on cerebral cortex of male Wistar rats. A single oral dose of Tributyltin-Chloride (TBTC) (10, 20, 30 mg/kg) was administered and the animals were sacrificed on day 3 and day 7. Blood–brain barrier permeability remained disrupted significantly till day 7 with all the doses of TBTC. Pro-oxidant metal levels (Fe, Cu) were increased with a concomitant decrease in Zn. ROS generation was substantially raised resulting in oxidative damage (increased protein carbonylation and lipid peroxidation) with marked decline in tissue antioxidant status (GSH/GSSG levels). Protein expression studies indicated astrocyte activation, upregulation of inflammatory molecules (IL-6, Cox-2 and NF-κB) and simultaneous elevation in the apoptotic index (Bax/Bcl2). Neurodegeneration was evident by reduced neurofilament expression and increased calpain cleaved Tau levels.

The *in-vitro* study demonstrated involvement of calcium and signaling molecules (p38), with downstream activation of caspase-3 and -8, and apoptotic cell death was evident by nuclear fragmentation, DNA laddering and Annexin V binding experiments. Ca^{2+} inhibitors (BAPTA-AM, EGTA, and RR) and free radical scavengers (NAC and biliprotein [C-PC]) increased cell viability (MTT assay), signifying specific roles of Ca^{2+} and ROS. Significance of p38 signaling was evaluated on pro-apoptotic proteins by using SB203580, a selective p38 inhibitor. Our data collectively illustrates that TBTC can disrupt BBB, induce oxidative stress, cause cell death and initiate neurodegeneration in rat brain.

1. Introduction

Tributyltin, a member of the organotin family, is extensively used as a biocide. It is now considered a global threat because of its widespread persistence in marine and fresh water environment (Elgethun et al., 2000; Kannan et al., 1996, 1999a; Strand and Jacobsen, 2005). Half life of tributyltin was found to be ~87 ± 17 years (Viglino et al., 2004). Human exposure occurs through TBT contaminated seafood and its use as fungicides on food crops. Additional exposure arises from the leaching of organotin-stabilized polyvinyl chloride used in food wrap, plastics and water pipes (Fent, 1996) and also via other sources like wine, household dust and food (Azenha and Vasconcelos, 2002; Fromme et al., 2005; Qunfang et al., 2004). TBT in the range of 50–400 nM was found in human blood samples in USA (Whalen et al., 1999) and in the range of 2.4–11 ng/g (0.007–0.033 nmol/g) in human liver in Poland (Kannan and Falandysz, 1997). TBT has been known to reduce cytotoxic activity of natural killer cells (Dudimah et al., 2007) and organ toxicities including neurotoxicity, cutaneous toxicity and hepatotoxicity (Snoeij et al., 1987). Various neurological complications have also been reported during occupational exposure to organotin (Yoo et al., 2007). Organotin antifoulants are banned in the EU, US and Japan since 1980s for small vessels (<25 m), following which a convention was adopted by the International Maritime Organisation (IMO). This prohibits the use of organotin on all vessels and offshore installations from January 2003. IMO further recommends that it should be removed or covered by a barrier to prevent leaching by January 2008 (ENDS Report, 2001; IMO, 2001).

Several reports indicate that redox imbalance plays a key role in metal catalyzed reactions primarily involving iron, copper and zinc in various neuropathologies (Gaeta and Hider, 2005). On the other hand calcium, a redox inactive metal, has significant effect on the blood–brain barrier (BBB) permeability (Brown and Davis, 2002). It has been speculated for long that various environmental factors are involved in the age related development of increased inflammation

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and oxidative damage to brain tissue. ROS can react with cellular macromolecules like proteins, lipids and DNA (Calabrese et al., 2000; Cardozo-Pelaez et al., 2000), leading to mitochondrial damage (Lin and Beal, 2006). Chemicals which increase ROS production and inflammation may certainly aggravate the situation and may act as a predisposing factor for neurodegenerative diseases.

In this regard, BBB integrity is vital in maintaining the microenvironment of brain tissue and its disruption is the initial event in several neurological diseases. Being lipophilic in nature, TBT was found to interact with the phospholipid bilayer of cell membranes (Ambrosini et al., 1996), which may lead to transient increase in BBB permeability (Hara et al., 1994). Other mode of TBT transport was reported in fish, where it reaches the brain tissue though axonal transport (Rouleau et al., 2003). TBT can alter the metabolism of various neurotransmitters (Tsunoda et al., 2004), affect neurobehaviour (Ema et al., 1991), decrease brain weight, and hinder synaptogenesis and myelinogenesis (Ocallaghan and Miller, 1988). Bo et al. (2011) showed that TBT can activate neuronal cells and Konno et al. (2005) demonstrated its effect on NMDA receptors in preweanling mice. Since TBT is demonstrated to reach the neuronal milieu, it is imperative to understand whether it influences the redox status in brain, thereby hampering the cellular microenvironment and altering cell survivability. We therefore, planned this study to evaluate whether TBT-Chloride (TBTC) can induce oxidative damage *in vivo* and also influence cell specific marker proteins accounting for cellular activity and viability. These were followed by *in vitro* experiments using dissociated mixed culture system to ascertain the mechanism of action of TBTC on cellular signaling, biochemical indices, mode of cell death and cellular susceptibility. We found that astrocytes were the primary target site of TBTC induced neurotoxic action. Calcium inhibitors and antioxidants exhibited protective efficacy, signifying critical role of calcium and ROS in neuronal cell death. p38 signaling was also found to be involved.

2. Materials and methods

2.1. Chemicals and reagents

Tributyltin chloride (TBTC), 2,4-dinitrophenylhydrazine (DNPH) and all the metal standard solutions was from Merck, Germany; 2-thiobarbituric acid was from MP Biomedicals, India; agarose, antibiotic–antimycotic solution, Dulbecco's phosphate buffer saline (PBS), propidium iodide (PI), papain, Hoechst 33258, sodium dodecyl sulphate, acrylamide, bis-acrylamide, Tris–HCl, MTT, BAPTA-AM, EGTA, ruthenium red, N-acetyl cysteine, Evans blue, dimethyl sulphoxide, bromophenol blue, proteinase-k, RNase-A, Tween 20, β-mercaptoethanol, formamide, N-ethyl maleimide, o-phthalaldehyde, Na_2-EDTA, protease inhibitor cocktail, glycerol, 10× Western blocking buffer, mouse anti-neurofilament (200 kDa) primary antibody, mouse anti-TAU primary antibody and all other chemicals were from Sigma–Aldrich, USA. Dulbecco's Modified Eagle's medium (DMEM-high glucose) was from Invitrogen, Life Technologies, USA; foetal bovine serum from Gibco BRL, Life Technologies, USA; rhodamine 123 (Rh123), 5′-chloromethylfluorescein diacetate (CMF-DA), 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) and Fluo-3 AM from Molecular Probes Inc., Eugene, Oregone; Annexin V-FITC reagent from Pharmingen (Becton Dickinson Company); rabbit anti-GFAP from Dako, Denmark; rabbit anti-Bax, rabbit anti-Bcl2, rabbit anti-IL-6 (M-19)-R, goat anti-caspase 3 (p17), rabbit anti-caspase 8 (p18), rabbit anti-glutathione-s-transferase (GST), rabbit anti-β actin, rabbit anti-metallothionein (Mt), rabbit anti-p-p38, goat anti-rabbit HRP conjugated secondary antibody and goat anti-mouse HRP conjugated secondary antibody from Santa Cruz, CA, USA; rabbit anti-Cox2, rabbit anti-p-ERK, rabbit anti-p-JNK were from Cell Signaling Technology, Beverly, MA, USA; PVDF membrane, rabbit anti-NF-κB (p-65) and rabbit anti-MBP primary antibody from Millipore, USA and SB203580 (p38 inhibitor) was from Calbiochem San Diego, CA, USA. Purified C-Phycocyanin (Grade-4) was from Delhi Neutraceuticals, New Delhi, India.

2.2. In-vivo experiments

2.2.1. Animals, treatment and tissue processing

A total of 100 male Wistar rats (4 week old) were procured from IITR animal house and maintained under standard conditions. Out of which, 80 rats were used in the *in-vivo* experiments and the rest for *in-vitro* studies. They were housed in plastic polypropylene cages (5 rats/cage) with a 12 h light/dark cycle and temperature of 25 ± 2 °C and were allowed to acclimatize for 1 week. They were fed with standard rodent pellet and water ad libitum. Our animal house and breeding facility are registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and CPCSEA guidelines were followed (IAEC approval obtained).

For *in-vivo* studies, a single dose of TBTC in corn oil (10, 20 and 30 mg/kg) was administered orally (1 ml/kg) at 11 o'clock to 20 rats/group and corn oil alone to control rats. Among each group, 10 rats were sacrificed on day 3 and the remaining 10 on day 7, with the day of dosing marked as Day 0 as shown in Electronic supplementary information (ESI) Fig. 1. On day 3 and day 7, 5 rats from each group were taken for BBB disruption studies and the remaining 5 animals were used for metal analysis along with biochemical measurements. Body weight of the animals sacrificed on day 7 (covering experimental period) was monitored daily to ascertain the effect of TBTC on body weight.

After sacrifice, whole brain was dissected, weighed and cortical tissue was taken for the following estimations. Approximately 100 mg tissue was put in acid mixture (HNO_3 and $HClO_4$, in 6:1 ratio) for metal analysis and rest of the tissue was snap frozen in liquid nitrogen and kept at −80 °C for determination of ROS, lipid peroxidation, protein carbonyl and GSH/GSSG. These estimations were carried out the following day. For ROS, TBARS and protein carbonyl, 10% homogenate was prepared in PBS and for GSH/GSSG, the homogenate was made in 0.1 M phosphate buffer containing 0.05 M Na_2EDTA. For protein expression studies, the tissue was homogenized in lysis buffer (50 mM Tris, 2 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% Tween 80, 1 mM DTT, 1 mM PMSF, 500 mM NaCl, 0.3% sodium orthovanadate, protease inhibitor cocktail), aliquoted and stored at −80 °C until analyzed. The expression levels of Bcl-2 family proteins (Bax, Bcl2), cell specific marker proteins (GFAP, NF, MBP), inflammatory mediators (NF-κB, IL-6 and Cox-2) and neurodegeneration marker (17 kDa TAU levels) were carried out within a week.

2.2.2. Quantification of BBB-disruption

This was done by using Evans blue dye as described by Gasche et al. (2001). Briefly, 2% Evans blue (3 ml/kg) in saline was injected though the tail vein 4 h prior to sacrifice. Animals were transcardially perfused for 45 min with ice cold 0.1 M PBS and the tissue was immediately weighed, homogenized in 500 μl formamide and left overnight at 37 °C. The homogenate was then centrifuged at 10,000 × *g* for 30 min and absorbance of the supernatant was read at 620 nm in a microplate reader (Fluostar Omega, BMG). The amount of Evans blue was determined from a standard curve plotted against known amount of the dye dissolved in formamide.

2.2.3. Metal analysis

Tissue was digested in acid mixture (HNO_3 and $HClO_4$ in 6:1 ratio) till tissue oxidation (sample turns white, but not to complete dryness). The final volume was made up to 1 ml with 1% HNO_3.

Tin (Sn) was determined at 284.0 nm in a Perkin Elmer HGA-400 graphite furnace coupled with Perkin-Elmer Model SIMAA 6000 atomic absorption spectrophotometer instrument equipped with AS-72 autosampler for delivery of samples and standard solutions. 0.1% palladium nitrate solution prepared in magnesium nitrate diluent was used as a modifier and argon as a source of neutral gas (Szoboszlai et al., 2001). Sn content in the tissue was calculated from a standard curve plotted using a tin solution (Merck). The amount of Sn value thus obtained was divided by the respective tissue weight and expressed as ng/g tissue.

Essential metals such as Fe, Cu, Zn and Ca were analyzed using atomic absorption spectrophotometer equipped with a vapor generation assembly (Varian AAS 250+ coupled with VGA 77; Varian Australia Pvt. Ltd) at 422.7 nm for Ca, 324.7 nm for Cu, 213.9 nm for Zn and 226 nm for Fe. Respective standards were used and the detection limit was 1.0 ppb (Behari and Prakash, 2006).

2.2.4. Generation of reactive oxygen species (ROS)

Generation of ROS was measured by using DCFH-DA (2,7-dichlorofluorescein diacetate). This fluorophore gets cleaved by esterases yielding DCFH, which further reacts with ROS to yield highly fluorescent DCF. 50 μl of tissue homogenate was diluted with 150 μl PBS. DCFH-DA dye was added (100 μM final concentration) and incubated at 37 °C for 15 min in dark (Rush et al., 2007). DCF fluorescence was read in a microplate reader (Fluostar Omega, BMG) at Ex: 485 nm and Em: 520 nm and the values expressed as fluorescence/mg protein.

2.2.5. Estimation of lipid peroxidation

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979). 50 μl tissue homogenate was mixed with 100 μl SDS (8.1%), 700 μl acetic acid (20%, pH 3.5), 700 μl TBA (0.8%), 450 μl of Milli Q water and then boiled for 15 min. After cooling at room temperature, it was centrifuged at 1500 × *g* for 15 min and the absorbance read at 532 nm in a microplate reader (Fluostar Omega, BMG). The levels were expressed as μmol/mg protein.

2.2.6. Protein carbonyl content

Oxidative damage to proteins is reflected by the amount of carbonyl groups incorporated in amino acid side chains by reactive oxygen free radicals. The carbonyl content was measured in brain tissue homogenates according to Cayman's protein carbonyl assay kit with minor modifications. Briefly, 50 μl sample homogenate