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Tributyltin chloride induced testicular toxicity by JNK and p38 activation, redox imbalance and cell death in sertoli-germ cell co-culture

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ABSTRACT

The widespread use of tributyltin (TBT) as biocides in antifouling paints and agricultural chemicals has led to environmental and marine pollution. Human exposure occurs mainly through TBT contaminated seafood and drinking water. It is a well known endocrine disruptor in mammals, but its molecular mechanism in testicular damage is largely unexplored. This study was therefore, designed to ascertain effects of tributyltin chloride (TBTC) on sertoli-germ cell co-culture in *ex-vivo* and in the testicular tissue *in-vivo* conditions. An initial Ca^{2+} rise followed by ROS generation and glutathione depletion resulted in oxidative damage and cell death. We observed p38 and JNK phosphorylation, stress proteins (Nrf2, MT and GST) induction and mitochondrial depolarization leading to caspase-3 activation. Prevention of TBTC reduced cell survival and cell death by Ca^{2+} inhibitors and free radical scavengers specify definitive role of Ca^{2+} and ROS. Sertoli cells were found to be more severely affected which in turn can hamper germ cells functionality. TBTC exposure *in-vivo* resulted in increased tin content in the testis with enhanced Evans blue leakage into the testicular tissue indicating blood–testis barrier disruption. Tesmin levels were significantly diminished and histopathological studies revealed marked tissue damage. Our data collectively indicates the toxic manifestations of TBTC on the male reproductive system and the mechanisms involved.

1. Introduction

The widespread use of tributyltin (TBT) as biocides in antifouling paints and agricultural chemicals has led to environmental and marine pollution. 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). TBT is best known as an endocrine disrupter in mammals (Keithly et al., 1999), increasing testosterone levels and causing imposex in female gastropod mollusks (Oberdorster and McClellan-Green, 2002). It can also cause sperm damage (McAllister and Kime, 2003) and induce development of male characteristics among females in some fish species (Shimasaki et al., 2003). Food chain accumulation and bio-concentration of organotin compounds is of prime concern for mammals. Trimethyltin (TMT), triethyltin (TET) and TBT show different organo-toxicities *in-vivo*. While TMT and TET induce strong neurotoxicity, TBT rather affects the immune system and is also reported to be teratogenic, developmental and reproductive toxicant (Nakanishi, 2008).

Studies in TBT induced reproductive toxicity regarding histological alteration, effect on fertility and spermatogenesis were reported previously. Decrease in sperm counts and vacuolization of sertoli cells in seminiferous tubules was shown by Kumasaka et al. (2002) using various doses of TBT (0.4, 2.0 and 10.0 mg/kg, p.o. for one month) in mice. In another study, apoptotic germ cells and inhibition of steroidogenesis was seen in mice after a single dose of TBT (25, 50 and 100 mg/kg) (Kim et al., 2008). It was also found to alter the hormone regulation, mainly testosterone (Ohno and Nakajima, 2005). A two generation reproductive study by Omura et al. (2001), demonstrated homogenization resistant spermatid count to decrease to 80% of control value in F1 generation by dietary concentration of 125 ppm TBT and in F2 generation by 25 and 125 ppm. Kishta et al. (2007) showed the appearance of lipid droplets in sertoli cells of neonatal rats upon *in-vivo* challenge with TBT, which may contribute to sertoli cell dysfunction and hamper spermatogenesis in rat testes. All these studies show the toxicological effect of TBT on the male reproductive system, but the underlying molecular mechanisms are still not well understood.

Over production of reactive oxygen species (ROS), which leads to oxidative stress, is deleterious to the cells of testicular tissue

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(Aitken and Roman, 2008). They cause oxidative injury to macromolecules such as lipids, proteins and DNA leading to cell death, either necrotic or apoptotic (Droge, 2002). Oxidative damage in rats by TBT has already been demonstrated in liver, thymus and brain tissue (Gupta et al., 2011; Ishihara et al., 2012; Mitra et al., 2013), however oxidative action of TBT in the testicular tissue is largely unexplored.

Since mammalian testicular cells are very susceptible to oxidative stress, and an impairment of spermatogenesis may result in infertility, we therefore, planned to appraise the toxicity of TBT on primary sertoli-germ cell co-culture, *ex-vivo*. Our aim was to delineate the various facets of TBT toxicity and the mechanisms involved therein. Critical role of calcium and ROS, in the mediation of cell death was thoroughly evaluated. The subsequent influence of TBTC on germ cell specific protein tesmin, apoptotic proteins along with cell signaling cascades involving MAPKinases were also considered. Effect of TBTC on testicular tissue *in-vivo* has also been addressed.

In the present investigation, we demonstrated induction of both apoptosis and necrosis in sertoli-germ cell co-culture by TBTC, following mitochondrial membrane depolarization via redox imbalance (ROS generation and depletion of GSH). Phosphorylation of JNK and p-38 kinases, indicate their involvement in downstream cascade of events.

2. Materials and methods

2.1. Chemicals used

All the chemicals were of highest grade purity available. Tributyltin chloride (TBTC) (purity >97%) and standard tin solution was purchased from Merck, Germany; Dulbecco's phosphate buffered saline (PBS), 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethidum bromide, agarose, n-acetyl cysteine (NAC), EGTA (ethylene glycol tetraacetic acid), BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid), bovine serum albumin (BSA), acrylamide, N,N′-methylene bis-acrylamide, N-N-N′-N′-tetramethyl ethylene diamine (TEMED), 10× western blocking buffer, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Hank's buffered salt solution (HBSS) and all other chemicals were purchased 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), Fluo 3-AM, Hoechst, Annexin V-FITC and 5′-chloromethylfluorescein diacetate (CMF-DA) from Molecular Probes inc, Eugene, Oregone; Propidium Iodide (PI) was from Calbiochem, San Diego, CA, USA; PVDF membrane was from Millipore, USA; and all the antibodies were purchased from Santa Cruz, CA, USA. All other chemicals used in the study were of analytical grade available in India. C-Phycocyanin (C-PC), Grade IV, was procured from Delhi Nutraceuticals Pvt. Ltd., New Delhi, India.

2.2. Ex-vivo study

2.2.1. Preparation of sertoli-germ cell co-culture

Male Wistar rats were procured and maintained in IITR animal house under standard conditions. They were housed in plastic polypropylene cages with a 12 h light/dark cycle and temperature of 25 ± 2 °C. 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).

Isolation of sertoli-germ cell co-culture as standardized in our lab (Khanna et al., 2011) was followed. Briefly, testes from 28 days old rat were dissected, decapsulated and teased in 10 ml HBSS. Cells were enzymatically released by treating with 1 mg/ml collagenase (type IV) for 30 min and then mechanically dissociated to obtain single cell suspension. The suspension was filtered through 30 μm nylon membrane and centrifuged at 80 × *g* for 10 min. The pellet obtained was washed with DMEM containing 10% FBS, resuspended and maintained in the same medium. The cell viability of this suspension comprising of sertoli and germ cells (30% and 70% respectively, counting based on morphological appearance) was assessed by tryphan blue exclusion test and was always found to be >95%. Asceptic procedures were followed throughout.

The co-culture in complete DMEM containing 10% FBS was seeded in well plates for 3 h and TBTC treatment was done subsequently.

2.2.2. Cell survival by MTT assay

Effect of TBTC on cell viability was determined by MTT assay (Mosmann, 1983). In light of the butyltin concentrations found in human blood (50–400 nM) (Whalen et al., 1999), we selected 300 nM as the lower dose (environmentally relevant) and subsequent higher doses (600 and 1000 nM) to study the effect of dose dependency on various cellular parameters. Sertoli-germ cell co-culture (1×10^4/well) were exposed to TBTC (300, 600 and 1000 nM) in 96-well plate, post seeding. TBTC was added in fresh DMEM without FBS for 6 and 24 h at 37 °C in a CO_2 incubator. 10 μl of MTT (5.4 mg/ml PBS) was added, 2 h prior to the completion time. After incubation, plates were centrifuged at 1200 × *g* for 10 min and supernatant was discarded. 200 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan and the absorbance was measured at 530 nm in a microplate reader (FluoStar Omega).

To delineate the role of Ca^{2+} inhibitors (50 μM BAPTA-AM, 5 mM EGTA) and free radical scavengers (50 μM NAC, 1 μg/ml CPC) on cell viability, the seeded cells were pre-incubated with them 1 h prior to TBTC treatment.

2.2.3. Microscopic observation of sertoli-germ cell co-culture morphology

Post seeding, 1×10^6 cells/well (12 well plate) were exposed to 600 nM TBTC in fresh DMEM without FBS and incubated for 6 h at 37 °C in a CO_2 incubator. To study the effect of Ca^{2+} inhibitors and free radical scavengers on cell morphology, the seeded cells were pre-incubated with them 1 h prior to TBTC addition. The cells were observed under light microscope at 200× magnification (Leica DMIL).

2.2.4. Assessment of apoptotic and necrotic cell population

Necrotic cell death is characterized by an early loss in cell membrane integrity. Whereas in apoptosis, although cell membrane integrity is maintained until the final execution of cell death, specific proteins (phosphatidylserine) are trafficked from inner to outer layer of the plasma-membrane to induce proper signaling pathways.

The mode of cell death induced by TBTC exposure was analyzed by using FITC conjugated Annexin V (specific for phosphatidyl serine) and PI (marker of cell membrane integrity loss) in a flow cytometer using Apoptosis detection kit (BD). Quadrants on Annexin V/PI dot plots was placed to denote live cells (lower left quadrant: Annexin V^-/PI^-), early/primary apoptotic cells (lower right quadrant: Annexin V^+/PI^-), late/secondary apoptotic cells (upper right quadrant: Annexin V^+/PI^+) and necrotic cells (upper left quadrant: Annexin V^-/PI^+) (Vermes et al., 1995). The total apoptotic population includes the percentage of cells in Annexin V^+/PI^- and Annexin V^+/PI^+ quadrant.

After seeding, 1×10^6 cells/ml were incubated with all the 3 concentrations of TBTC for 6 and 18 h and then suspended in 500 μl annexin binding buffer (1×). 5 μl Annexin V-FITC and 10 μl PI were added and incubated for 30 min in dark at RT. FITC and PI fluorescence of 10,000 events were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm), respectively, on BD-LSR flow cytometer and the data analyzed using Cell Quest software.

For ascertaining the effect of Ca^{2+} inhibitors and free radical scavengers on cell death, they were added 1 h prior to 600 nM TBTC treatment and further incubated for 6 h.

2.2.5. DNA fragmentation

During apoptosis, endonucleases cleave DNA into several fragments of 180 base pairs and its multiples, which give a laddering appearance when run on agarose gel. To confirm the role of TBTC in inducing apoptosis, sertoli-germ cell co-culture (3×10^6/well) were exposed to various concentrations of TBTC for 6 h. Total genomic DNA was isolated by treating the cells with Proteinase-k for 2 h (37 °C) and then with RNase for 4 h (56 °C) followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction. Isolated DNA was dried, dissolved in 1X TBE buffer, quantified in NANODROP spectrophotometer and 2 μg DNA was loaded onto 1.5% agarose gel. DNA was stained with ethidium bromide (0.5 μg/ml) for 15 min and the bands visualized under UV illumination in a Gel Documentation system (Syngene GBox HR-16).

2.2.6. Calcium measurement

Post seeding, sertoli-germ cell co-culture (1×10^6/ml) were suspended in physiological HEPES-buffered solution containing 130 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM HEPES Buffer (pH 7.4) and preloaded with 4 mM Fluo-3AM for 30 min. TBTC (600 nM) was then added and Fluo-3 fluorescence was monitored at 525 nm in a microplate reader from 0 to 180 min, according to the modifications of Lyng et al. (2000).

2.2.7. Generation of reactive oxygen species

2′,7′-Dichlorofluorescein diacetate (DCFH-DA), a lipophilic dye is deacetylated by cellular esterases to non fluorescent dye DCFH, which is then oxidized to DCF and this gives a quantifiable measure of the cellular oxidant production (Zamzami et al., 1995).

Post seeding, sertoli-germ cell co-culture (1×10^6/ml) were exposed to different concentrations of TBTC for 1, 3 and 6 h. DCFH-DA (100 μM) was loaded onto the cells for 30 min in dark at 37 °C, prior to the completion time. Cells were then washed, re-suspended in 200 μl PBS and fluorescence was measured in a microplate reader (FluoStar Omega) with Ex: 488 nm and Em: 520 nm.

2.2.8. Glutathione estimation

5′-Chloromethylfluorescein diacetate (CMF-DA) a selective probe for GSH, was used for quantification of intracellular glutathione content (Okada et al., 2000). Post seeding, 1×10^6 cells/ml were exposed to all the three concentrations of TBTC for

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