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RESEARCH ARTICLE

Impact of tributyltin on antioxidant and DNA damage response in spermatozoa of freshwater prawn *Macrobrachium rosenbergii*

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Abstract Effects of tributyltin (TBT) on antioxidant [total superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR)] and DNA damage levels in the spermatozoa were studied and reported here for the first time in the freshwater prawn *Macrobrachium rosenbergii*. Three groups of (n=10 in each group) fishes were exposed to three different nominal concentrations of TBT viz., 1, 2, and 4 mg L^{-1} along with control group for 90 days. Significant decrease of antioxidant and increased DNA damage levels were seen at higher doses of 2 and 4 mg L^{-1}. In prawn, the antioxidant level plays a vital role in sperm protection, activation, differential functions related to the physiology, and reproductive behavior. This study serves as a biomonitoring tool to assess the TBT effects on reproductive behavior of aquatic biota.

Keywords TBT · Spermatozoa · SOD · GPx: GR · Comet assay

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Introduction

Recently, tributyltin (TBT) compounds have gained prominence due to its endocrine disruptive property. During the past few decades, the scientists especially ecotoxicologists have been concerned with new type of pollutants associated with antifouling paints known as organotin compounds (Meador 1997; Gooding et al. 1999; Hall et al. 2000; Amr 2004). Among these, the most prominent are tri-substituted forms, such as tributyltin (TBT) and triphenyltin (TPhT). TBT has been used for several years in marine coatings (Harrison 1989; Rudel 2003; Merian et al. 2004; Catharios et al. 2014) and also used as stabilizers in plastics, pesticide control of schistosomiasis, and antifungal action in textiles and industrial water systems (Amr 2004).

TBT is highly toxic to many aquatic organisms and is still detected in aquatic ecosystems even at low concentrations (Fent 1996; Alzieu 2000; Rudel 2003). Effects of TBT have been investigated in several aquatic organisms, including algae (Waldock and Thain 1983) and crustaceans (Laughlin et al. 1983; Bushong et al. 1988; Pinkney et al. 1990). Many studies have shown the effect of TBT on growth (Fent and Meier 1994; Bentivegna and Piatkowski 1998) and reproduction (Nirmala et al. 1999; Inoue et al. 2004; Nakayama et al. 2005; Shimasaki et al. 2006).

Antioxidant plays an important role in sperm motility, integrity, metabolism, and function, protecting the cells against oxidative damage (Alvarez and Storey 1983). Their effects have been widely studied in mammalian spermatozoa (Aitken and Baker 2004); however, a limited amount of information on the precise mechanism on the action of antioxidant systems in fish sperm is available.

Reactive oxygen species (ROS), a class of pro-oxidants derived from O_2 that naturally occur as a result of oxidative metabolism, can damage macromolecules when left

unquenched by the antioxidant system (Halliwell and Gutteridge 1985). Sperms are particularly susceptible to the attack by ROS, owing to their limited antioxidant machinery, their high metabolic activity, and high polyunsaturated fatty acid (PUFA) content of their membranes (Sikka 2001; Surai et al. 1998, 2001; Tremellen 2008). Oxidative insult to sperm can damage the mitochondria, the cytoskeleton, and the sperm axoneme, resulting in a reduction of energy availability and sperm motility (MacLeod 1943; Jones et al. 1979; Wishart 1984; de Lamirande and Gagnon, 1992; de Lamirande et al. 1997, 1998).

The abnormalities of sperm chromatin structure and DNA fragmentation due to environmental conditions have been identified by different techniques in different species (Evenson et al. 1980; Duke and Cohen 1986; Ballachey et al. 1987; Evenson et al. 1994; Aravindan et al. 1997; Homma-Takeda et al. 2001). There are different methods to determine the DNA fragmentation, such as the conventional agarose gel electrophoresis and single-cell gel electrophoresis (SCGE). The "microelectrophoretic study" of DNA damage in individual cells was first described by Ostling and Johanson (1984). This single-cell gel electrophoresis technique has been modified and extensively validated, and now, it is commonly referred to as the "comet" assay (Collins 2004). The alkaline comet assay was firstly described by Singh et al. (1988). This assay follows the process of cell lysis and DNA unwinding; the DNA fragments flow from the nucleus and move to the positive pole, generating a comet-like band. After fluorescent staining, DNA damage of the cells can be observed under the microscope. The fluorescence intensity of the comet tail depends on the degree of DNA damage. The degree of DNA damage is one of the most important indicators of sperm quality, high sensibility, fast, and straightforward. SCGE is widely used for the detection of DNA damage in plant cells (Koppen et al. 1999; Maluf and Erdtmann 2000; Gi and Richard 2004), mice cells (Giovannelli et al. 2003), lymphocytes (Stavreva et al. 1998), fish sperm (Labbe et al. 2001; Zilli et al. 2003; Xu et al. 2005), and human sperm (Xu et al. 2000; Lu et al. 2002).

The aim of this present study is to focus on the antioxidant parameters and DNA damage levels in the spermatozoa of freshwater prawn *Macrobrachium rosenbergii* exposed with TBT. Antioxidants are important for the protection and activation of spermatozoa, regulates all other biochemical parameters, and protects the structure of sperm DNA.

Material and methods

Experimental prawn specimens

Mature adult male *M. rosenbergii* weighed between 100 and 150 g of standard length 17±2.0 cm were procured from a commercial farm and transported to the laboratory in oxygenated bags and released into 200 L fiber tank (10 prawns per tank) filled with dechlorinated tap water. They were then acclimatized for 21 days under laboratory conditions with natural photoperiod and fed with commercial feed. The fecal matter and other waste materials were siphoned off daily to reduce ammonia content in water that was renewed once in 2 days with dechlorinated tap water. The water quality parameters were analyzed and maintained within the normal range (pH 7.5±0.9, dissolved oxygen 8.2±1.2 mg L^{-1}, temperature 25±1.7 °C, and hardness—in terms of $CaCO_3$ was 220 mg L^{-1}).

Collection of milt

After 90 days of experimental period, the milt samples were obtained from matured *M. rosenbergii* male prawns by stripping the abdomen of each prawn. The initial male ejaculate was discarded, and the external urinogenital pore was wiped dry with paper towel to avoid contamination from seawater, urine fecal matter, and hemolymph. Collected milt samples were provided enough oxygenation to the sperm by keeping enough head space in the tubes.

Assessment of antioxidant parameters

Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974) involving the autoxidation of pyrogallol and was assessed spectrophotometrically at 420 nm. Glutathione peroxidase (GPx) activity was assayed, based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with glutathione reductase (GR). The specific activity was determined using the extinction coefficient of 6.22 mM cm^{-1} (Lawrence and Burk 1976). GR activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg and Mannervik 1975). One unit of SOD activity is defined as the amount of the enzyme needed to effect 50 % dismutation of superoxide radical per minute. One unit of GPx or GR activity is defined as the amount of the enzyme that consumes about 1 μmol of substrate or generates 1 μmol of product per min. Activity was expressed as international milliunits (mU) per milligram of protein. All parameters of antioxidant activity were made in triplicate for each sample.

Assessment of DNA damage

The single-cell gel electrophoresis (comet) assay was performed on the protocol described by Li et al. (2008a). Molecular grade and DNAse-free reagents (Sigma Aldrich, USA) were used throughout the experiment. Microscopic slides were used for comet assay, and each slide was prepared in the following manner: 50 μL of sperm (6×106 cells mL^{-1}) were diluted in 5 mL of phosphate-buffered solution (PBS). Diluted samples (200 μL) were mixed with 700 μL of 0.8 %

Nu Sieve GTG low-melting temperature agarose. Finally, 50 μL of this mixture was added to the slide and allowed to solidify for1 h.

After 1 h, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10 % dimethyl sulfoxide, and 1 % Triton X-100, pH 10). The slides were treated with proteinase K (20 mM Tris-HCl, 1 mM $CaCl_2$, and 50 % glycerol, pH 7.4) and incubated overnight at 32 °C (Li et al. 2008). After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with alkaline buffer (300 mM sodium hydroxide, 1 mM EDTA, pH 12.3) for 20 min to allow the DNA to unwind. The buffer level was adjusted to approximately 0.25 cm above the slides, and electrophoresis was carried out for 20 min at 35 V and 170 mA. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. For comet visualization, 50 μL of Vista Green DNA Staining Solution was loaded onto the slides that were subsequently covered with a cover-slip and analyzed with an Olympus BX50 fluorescence microscope at 20× magnifications. A total of 100 cells were scored for each sample, and the captured images were analyzed using comet score image analysis software. Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (% tail DNA) were measured. Olive tail moment (tail length× tail DNA content) was calculated using the following formula:

$$M_{\mathbf{Tail\ Olive}} = (\mathbf{CG_{Tail}} - \mathbf{CG_{Head}})\ \mathbf{x}\ \%\ \mathbf{DNA_{Tail}} \qquad (1)$$

where$M_{\text{Tail Olive}}$ is the Olive tail movement, CG_{Tail} the center of the gravity of the tail, CG_{Head} the center of gravity of the head, and % DNA in the tail compared to the head were determined.

Results and discussion

The results of the antioxidant [SOD, GPx, and GR] and the DNA damage levels in the spermatozoa of freshwater prawn *M. rosenbergii*, control, and TBT-treated groups were shown in Figs. 1, 2, 3, and 4. The results demonstrated that the antioxidant defense levels in spermatozoa of freshwater prawn *M. rosenbergii* decreased with increasing in the dose levels of TBT. At lower dose of about 1 mg L^{-1}, it did not show a significant decrease of the antioxidant compared with control groups. Significant decreases in these antioxidants were seen at higher doses of 2 and 4 mg L^{-1}. SOD, GPx, and GR levels in sperm samples decreases about 85, 92, and 90 %, respectively, at 4 mg L^{-1}exposure group.

Spermatozoa DNA damage was assessed by comet assay method. DNA damage were analyzed with comet score 1.5 software to obtain parameters such as comet length, tail length, and percentage DNA in the tail. A significantly higher percentage of tail DNA was found in spermatozoa of 4 mg L^{-1}exposure group. In the higher doses like 2 and 4 mg L^{-1} groups, the antioxidant levels were decreased, and simultaneously, ROS levels were increased which led to severe DNA damage in the spermatozoa. This difference in ROS relative to the antioxidant system of spermatozoa can potentially result in metabolic or functional disorders and reduction in sperm motility (Li et al. 2009). The relatively high polyunsaturated fatty acid content of the spermatozoon membrane makes it susceptible to oxidative damage (Trenzado et al. 2006).

Fish sperm possesses an antioxidant system that consists of enzymatic (glutathione peroxidase and reductase, catalase, superoxide dismutase) and non-enzymatic (α-tocopherol, ascorbic acid, β-carotene, selenium, zinc) components that are capable of counteracting with the damaging effects of ROS and protect the cell structure (Li et al. 2009). However, a limited amount of information is available about the precise

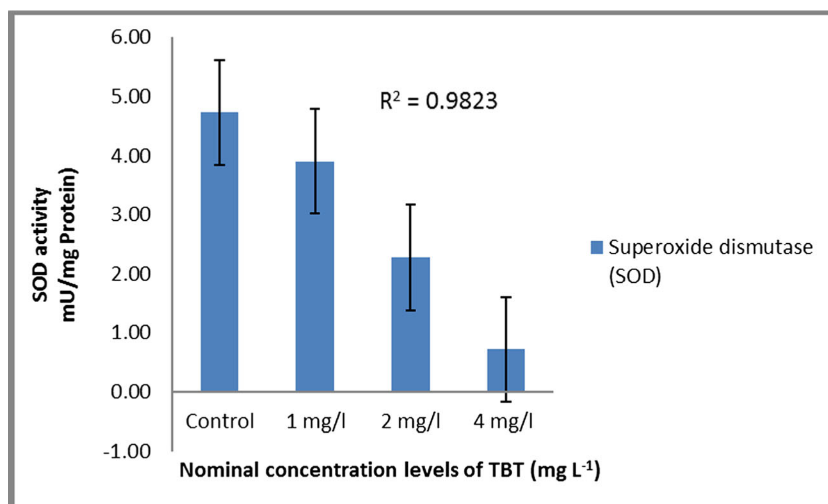

Fig. 1 Results of superoxide dismutase activity on spermatozoa of freshwater prawn *M. rosenbergii*, control, and TBT-treated groups

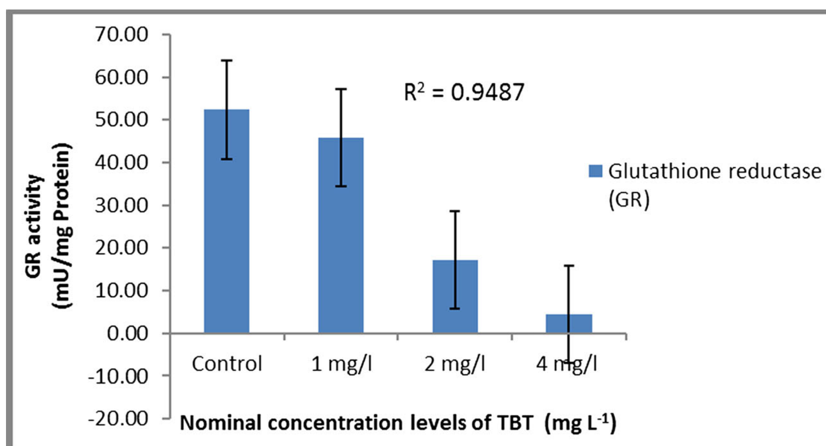

Fig. 2 Results of glutathione peroxidase activity on spermatozoa of freshwater prawn *M. rosenbergii*, control, and TBT-treated groups

mechanism on the action of antioxidant systems in fish sperm (Lahnsteiner et al. 2010).

Lipid peroxidation can lead to loss of membrane integrity, causing increased cell permeability, enzyme inactivation, resistance to osmotic shock, and decrease in fertilization potential. In addition, protein oxidization leads to deleterious effects on sperm function, with plasma membrane structure proteins, as well as proteins having enzyme activity, being affected (Domínguez-Rebolledo et al. 2010; Shaliutina et al. 2013). Lipid peroxidation is particularly important for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids compared with other species. Lipid peroxidation has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey 1996).

The major role of antioxidants is implied in the inactivation or transformation of oxidants, which can be either transformed by antioxidant enzymes into less reactive forms or can react with antioxidant molecules that are chemically stable. In the current study, the antioxidant activity was evaluated as total SOD activity, GPx, and GR activity. Superoxide dismutase plays a major role in decreasing LPO and protecting spermatozoa against oxidative damage (Sikka 1996). In contrast, several studies reported that SOD is present in high amounts in seminal plasma of all mammalian species, apparently to protect spermatozoa against oxidative stress, and it is also one of the major factors that prevent premature capacitation (de Lamirande et al. 1993; Cassani et al. 2005). Therefore, we can conclude that reduced SOD activity in fish seminal plasma might result in reduced protection against ROS.

Glutathione peroxidase, a selenium-containing antioxidant enzyme, removes peroxyl radicals from various peroxides, including H_2O_2, whereas GR regenerates reduced glutathione from its oxidative form (Sikka 2001). The enzyme system comprising GR provides defense mechanism against lipid peroxidation in mammalian sperm, and defects in activity of this enzyme can lead to a loss of cell function (Cheema et al. 2009).

This study represents the first successful attempt to apply this assay to detect DNA damage in prawn sperm and relate this to reproduction. DNA damage, as indicated by comet assay, showed a clear dose-dependent response to TBT and associated with enhanced ROS production. Mature

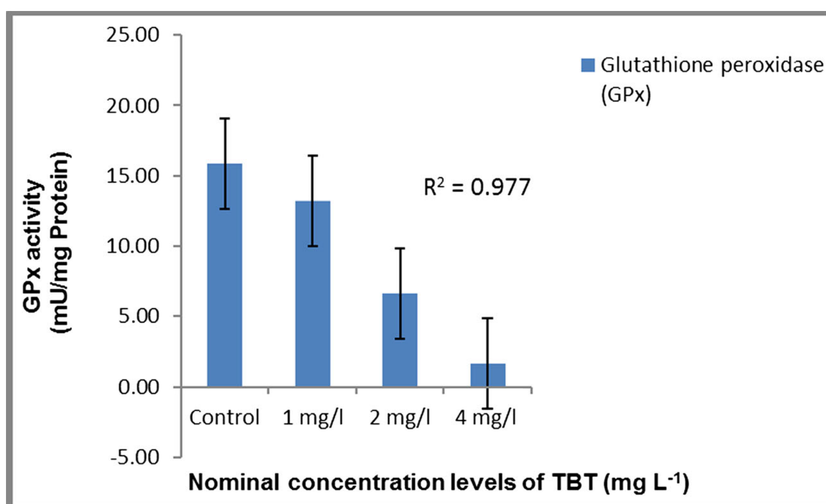

Fig. 3 Results of glutathione reductase activity on spermatozoa of freshwater prawn *M. rosenbergii*, control, and TBT-treated groups

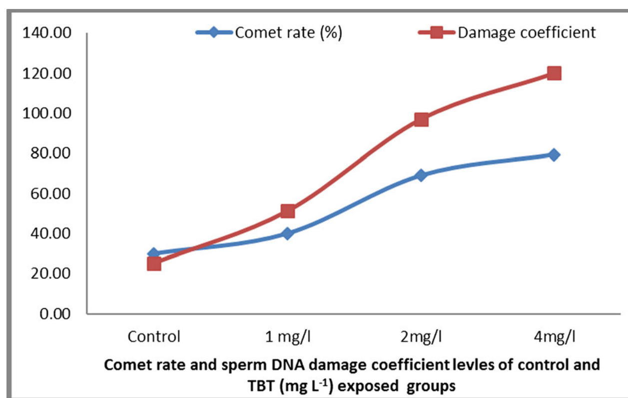

Fig. 4 Comet rate and sperm DNA damage coefficient levels of control and TBT-exposed groups

spermatozoa has a low DNA repairing ability and also a weak antioxidant capacity (Van Loon et al. 1991) and is therefore more prone to oxidative stress caused by ROS.

ROS production and the resultant lipid and DNA damages have been implicated in detrimental effects on reproduction (Agarwal et al. 2003). In mammals, ROS itself has been shown to inhibit sperm movement via the depletion of intracellular ATP (Armstrong et al. 1999). Studies have also indicated that oxidative damage to sperm plasma membrane by ROS may impair sperm function, leading to male infertility (Agarwal et al. 2003; Zhou et al. 2006). In addition, sperm DNA quality is vital for correct conveyance of genetic material to the next generation, and negative correlations have been recorded between the percentage of spermatozoa with fragmented DNA and the decline in sperm counts associated with male infertility (Sun et al. 1997; Shen et al. 1999; Barroso et al. 2000). Conceivably, the marked increase in sperm ROS production associated with DNA damage and significant decrease in sperm motility may, in turn, impair prawn reproduction. Revathi et al. 2014 reported that the TBT with three concentration levels such as 10, 100, and 1000 ng L^{-1} clearly manifests the deleterious effects on spermatogenesis, reduction in testosterone level, and impairment in the reproductive system of freshwater male prawn, *M. rosenbergii*. In another study, from the same dose levels of TBT administration has remarkable impact on the testicular development, impairment of spermatogenesis as well as decrease of testosterone level in the freshwater male prawn, *M. rosenbergii* (Revathi et al. 2012). The present results agree with the aforementioned observations, and this study clearly indicates the impact of TBT on the antioxidant and DNA damage response in spermatozoa of freshwater prawn *M. rosenbergii*.

Conclusion

As far as our knowledge is concerned, this is the first study focusing on the effects of antioxidant and DNA damage levels in the spermatozoa of freshwater prawn *M. rosenbergii* exposed to three different dose levels of TBT under laboratory conditions. This investigation has identified significant decreases of the sperm antioxidants and DNA damage at higher doses such as 2 and 4 mg L^{-1}. The present study suggests that the ROS levels increased with high doses and it leads to tremendous decrease in the levels of sperm antioxidants and also causes severe DNA damage in male prawns. This biomonitoring study is going to be useful in assessing the TBT effects on the reproductive behavior of aquatic biota.

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