The Effect of In Vitro Exposure to Tributyltin on the Immune Competence of Chinook Salmon (*Oncorhynchus tshawytscha*) Leukocytes

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Abstract We evaluated the direct effects of in vitro exposures to tributyltin (TBT), a widely used biocide, on the cell-mediated immune system of Chinook salmon (*Oncorhynchus tshawytscha*). Splenic and pronephric leukocytes isolated from juvenile Chinook salmon were exposed to TBT (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/l) in cell cultures for 24 h. Effects of TBT on cell viability, induction of apoptosis, and mitogenic responses were measured by flow cytometry. Splenic and pronephric leukocytes in the presence of TBT experienced a concentration-dependent decrease in viability in cell cultures. Apoptosis was detected as one of the mechanisms of cell death after TBT exposure. In addition, pronephric lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocytes. The functional ability of splenic B-cells to undergo blastogenesis upon lipopolysaccharide stimulation was also significantly inhibited in the presence of 0.05, 0.07, or 0.10 mg/l of TBT in the cell cultures. Flow cytometric assay using a fluorescent conjugated monoclonal antibody against salmon surface immunoglobulin was employed for the conclusive identification of B-cells in the Chinook salmon leukocytes. Our findings suggest that adverse effects of TBT on the function or development of fish immune systems could lead to an increase in disease susceptibility and its subsequent ecological implications.

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Tributyltin (TBT) has been widely used as an agricultural fungicide and antifouling paint for vessels and, as a result, has been released into aquatic environment since the 1960s (Fent 1996). Although the use of TBT paints has been banned recently (Champ 2000), significant quantities of TBT and its metabolites are still detectable in many regions (Berto et al. 2007; Viglino et al. 2004). They can persist for prolonged periods in aquatic sediment and aquatic organisms (Chau et al. 1997; Kannan and Falandysz 1997; Strand and Jacobsen 2005). A number of studies have reported that TBT exposure causes serious environmental and health problems in nontarget aquatic organisms (Axiak et al. 2000; Matthiessen and Gibbs 1998; Short and Thrower 1986; Sumpter 1998). Currently, TBT has been recognized as one of the most toxic anthropogenic chemicals released into the aquatic environment (Mee and Fowler 1991). TBT is known to alter the immune response in fish; for example, Grinwis et al. (2000) concluded that the high prevalence of lymphocystis virus infections in fish observed in field studies could be related to the TBT exposure. In vivo exposures to TBT under controlled laboratory conditions resulted in a variety of effects on fish immune systems including thymus atrophy, the reduction

of circulating lymphocytes (Schwaiger et al. 1992), and the depletion of mitogenic responses (Harford et al. 2007). In contrast, similar studies demonstrated an elevation of circulating granulocytes (Schwaiger et al. 1994; Wester and Canton 1987) and the stimulation of phagocytic function (Harford et al. 2007).

The Chinook salmon (Oncorhynchus tshawytscha) is a commercially valuable anadromous fish that remains in estuaries, preying on benthic invertebrates, before migrating from the river into the ocean. Marine fish and diadromous fish have higher exposure rise to TBT than fresh water fish (Ohji et al. 2006) and those fish that remain in highly contaminated urban estuaries have a higher incidence of infectious diseases (Arkoosh et al. 2001; McCain 1991; Watermann and Kranz 1992). Anthropogenic compounds clearly alter the function or development of fish immune systems (Anderson et al. 1984; Arkoosh et al. 1996; Carlson et al. 2002). However, very little research has been conducted on the immune response of juvenile salmon to TBT exposure.

The present study was undertaken to examine the toxicity of TBT on the splenic and pronephric leukocytes isolated from Chinook salmon under in vitro conditions. The immunotoxicity was assessed using cell viability, apoptosis, and lymphocyte proliferation assays. Cytotoxicity is generally considered as the potential of a substance to induce cell death. Most past studies examining cytotoxicity of TBT in fish leukocytes measured necrosis. However, apoptosis is also an equally important mechanism of cell death for evaluating immunotoxicity (Alison and Sarraf 1995). Moreover, there is particular interest in the differential cytotoxic sensitivity in fish leukocytes, because the polarization of some specific leukocytes following in vivo exposures to TBT has been reported (Nakayama et al. 2007; Schwaiger et al. 1994; Tada-Oikawa et al. 2008). Finally, the mitogenic stimulation assay was carried out to determine the effect of TBT exposure on lymphocytes proliferation. The mitogenic cellular responses are potentially sensitive indicators commonly used to evaluate immunotoxicity of exposure to xenobiotics on lymphocyte function (Arkoosh et al. 1996; Faisal et al. 1991). Although a suppression of mitogenic responses of lymphocytes following in vivo exposures to TBT has been reported by some researchers (Harford et al. 2007; Rice et al. 1995), very little is known about the direct toxic actions of TBT on fish humoral immunity mediated by B-cells. Additional laboratory experiments using more precise techniques such as an in vitro lymphocyte cell culture system coupled with a sensitive assay such as the flow cytometric assay are needed to fully evaluate and explain the cause-and-effect relationships between TBT exposure and the B-cell-mediated humoral immunosuppression in fish.

Materials and Methods

Fish

Yearling spring Chinook salmon (Marion Forks stock) weighing 127.46 ± 7.30 g [mean $\pm$ standard error of mean (SEM)] were housed at the Fish Performance and Genetics Laboratory, Oregon State University, Corvallis, Oregon. The fish were maintained in 0.9-m circular fiberglass tanks supplied with 12–13°C flow-through water under natural photoperiod and fed a commercial diet of Semi-Moist Pellets (BioOregon[TM], Warrenton, OR) twice daily.

Chemicals and Reagents

Tissue culture medium (TCM) contained 7% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 1% L-glutamine (Sigma), 200 IU/ml penicillin (Sigma), and 0.2 mg/ml streptomycin (Sigma) in Minimum Essential Media (MEM; Invitrogen Co., Carlsbad, CA) buffered with sodium bicarbonate. Tri-n-butyltin methoxide (TBT, 97% purity; Sigma) was dissolved in 100% ethanol and diluted to working solution in TCM for no more than 2 h before starting the incubation with cells. The final concentration of ethanol in each TBT-treated culture and vehicle control was always 0.003%. Lipopolysaccharide (LPS; Sigma) from Escherichia coli serotype O 55:B5 was dissolved in the TCM to a final concentration of 200 μg/ml in each cell culture. Isolation medium was composed of Hank's balanced salt solution and Alsever's solution (0.1 M dextrose, 70 mM sodium chloride, and 30 mM sodium citrate).

Tissue Sample

Fish were rapidly netted from their tanks, immediately killed in 200 mg/l buffered tricaine methane-sulfonate (MS 222), weighed, and then bled by caudal severance. The fish were then transported on ice to our immunology laboratory at Oregon State University within 30 min. The spleen and pronephros were aseptically isolated and placed separately into individual conical tubes filled with 1 ml of cold isolation medium.

Isolation and Cultivation of Leukocytes

The isolation of leukocytes from spleen and pronephros was performed according to the method of Crippen et al. (2001). The isolated tissue was placed on a 40-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) and gently disrupted with the end of a 3 ml syringe plunger. The disrupted tissue was washed through a strainer into a

50-ml polypropylene conical tube (Becton Dickinson) with isolation medium. The homogenized tissue suspension was then centrifuged at $500g$ for 7 min at 4°C and the supernatant was aspirated. The pellet was resuspended with 2 ml of ice-cold isolation medium, and clumps were removed. Hypotonic lysis was used to purify and separate leukocytes from erythrocytes. Briefly, the 2 ml of cell suspension were diluted with 9 ml sterile deionized water to lyse the erythrocytes for 20 s; then 1 ml of sterile $10\times$ phosphate-buffered saline (PBS) was immediately added to stop lysing. Cells were washed twice by centrifugation at $500g$ for 7 min at 4°C, the supernatant aspirated, and the cells resuspended in 2 ml of ice-cold TCM. After the purification of leukocytes, viable cells were counted using a Trypan blue dye exclusion test, and the cell suspension was diluted with ice-cold TCM to final concentration of 5×10^6 viable cells/ml. Cell suspensions for each individual fish were kept separate. The cell suspension (100 μl/well) at a concentration of 5×10^6 viable leukocytes was plated out into flat-bottom 96-well plates (Becton Dickinson); then TBT solution, LPS solution, and/or TCM were added to a final volume of 200 μl/well. The cell cultures were maintained under dark condition at 17°C in an incubator culture chamber (C.B.S. Scientific CO., Del Mar, CA) containing blood gas mixture (10% O_2, 10% CO_2, and 80% N_2) throughout this study (Miller and McKinney 1994), unless otherwise indicated.

Flow Cytometric Assay of Cell Viability

Splenic and pronephric leukocytic cells in culture were exposed to various concentrations of TBT (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/l) for 24 h. TCM alone control was a 1:1 mixture of cell suspension and TCM. Vehicle control was a 1:1 mixture of cell suspension and TCM containing ethanol (0.006%). Ten microliters of propidium iodide solution (PI; 50 μg/ml in PBS) were added into each well of the 96-well plate, and cells were incubated for 10 min at room temperature. After incubation, cell viability was analyzed by flow cytometry (FACScan®; Becton Dickinson), as described previously (Misumi et al., 2005). Cells that stained negative for PI were determined as viable cells, and PI positive cells were determined as dead cells and excluded from further analysis. The percentage of PI negative cells in total leucocytes was calculated using the software program, Cell Quest (Becton Dickinson).

Flow cytometry was also used to distinguish between lymphocytes, granulocytes, and other cells in the pronephric leukocyte suspension based on cell size and granularity, as described previously (Misumi et al. 2005). Analysis of forward (a measure for cell size) and side (a measure for cell granularity, complexity) scatter patterns indicated one major population in the splenic cell suspension and two major populations in the pronephric cell suspension. Those cell populations were identified as lymphocytes and granulocytes based on microscopic analysis with Wright–Giemsa staining using the Hema 3 stain set (Biochemical Sciences, Inc., Swedesboro, NJ) following isolation of populations using a cell sorter (MoFlo®, Cytomation, Inc., Fort Collins, CO).

Flow Cytometric Assay of Apoptosis

To investigate the possible involvement of apoptosis in TBT-mediated inhibition of cell viability, flow cytometric analysis was performed. Splenic and pronephric leukocyte cell cultures were exposed to TBT (0, 0.3, and 0.6 mg/l) for 6 h. The percentage of apoptotic cells (Annexin V positive and PI negative) was measured using an ApoAlert Annexin V-FITC Apoptosis kit (BD Biosciences Clontech, Palo Alto, CA), with flow cytometry as previously described (Misumi et al. 2005). Control cultures contained 0.003% ethanol as a vehicle control. Following incubation, 50 μl of the cell suspensions were centrifuged in microtubes (Bio-Rad Laboratories, Inc., Hercules, CA) at $500g$ for 7 min at 4°C and resuspended in 40 μl of Annexin V Binding Buffer containing 1 μl of Annexin V-FITC and 2 μl of PI. Cells were incubated for 10 min at room temperature according to the manufacture's protocol (ApoAlert Annexin V-FITC Apoptosis kit; BD Biosciences Clontech). Apoptotic cells (Annexin V positive and PI negative) were detected and distinguished by flow cytometry.

Flow Cytometric Assay of Mitogenic Response

This experiment was performed to determine if TBT suppressed the blastogenesis of surface IgM presenting leukocytes (B-cells) isolated from the spleen and pronephros. The flow cytometric assay for B-cell blastogenesis measuring functional humoral immunocompetence in Chinook salmon was previously described by Milston et al. (2003) and Misumi et al. (2005). Splenic and pronephric leukocytes were exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg/l) with or without LPS for 96 h. TBT and LPS were present for the whole duration in the cell culture. Flow cytometry was used for measuring the percentage of Ig + blasting (larger) B-cells in the viable cell population. Biotin-labeled anti-salmon and trout Ig Mab solutions were purchased from DiagXotics Inc. (Wilton, CT) and used to detect B-cells expressing Ig on their cell surface. At same time, cell viability was also measured to determine if cell death might be related to the inhibition of blastogenesis by TBT.

Statistical Analysis

The mean and SEM were determined for nine individual fish samples ($N = 9$) for each immune test. There was no replication of assays for any individual fish. Percent values were transformed by the arcsine of the square root of the value for further statistical analysis. Parametric statistical tests (the Student's t-test and ANOVA) and nonparametric statistical tests (the Kruskal–Wallace test and the Mann–Whitney U-test) were used for statistical comparisons of data. Measurements were considered significant when p-values of both parametric and nonparametric tests were below 0.05. Tukey's honestly significant difference procedure (HSD) was used to determine which treatments were significantly different from others.

Results

Cell Viability

Exposure of Chinook salmon leukocytes to TBT resulted in decreased vialibity of these cells in culture and the decrease in viability was directly correlated to the concentration of TBT (Fig. 1). There was no significant difference between splenic and pronephric leukocytes in their response to TBT; and there was no difference in cell viability between cells cultured in the control medium alone or in the vehicle control medium (data not shown).
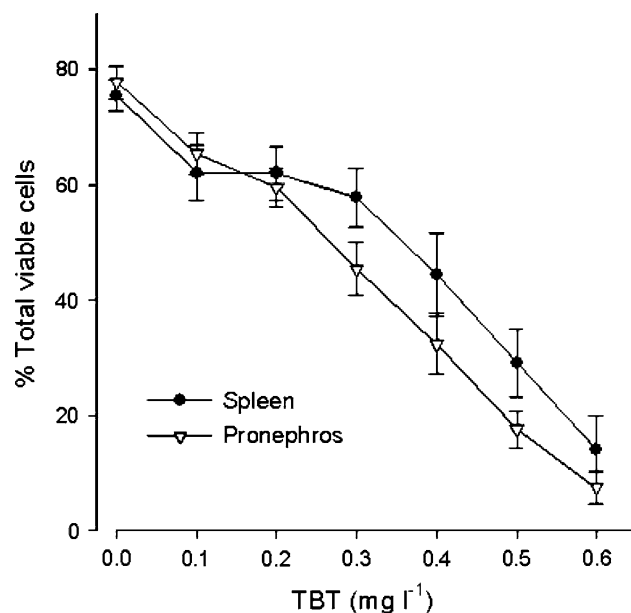
Apoptosis

The primary cause of cell death with TBT treatment was determined by testing the treated cells for apoptotic markers. In this study, we used FITC-conjugated Annexin V, which detects one of the earliest signs of apoptosis: the translocation of phosphatidylserine from the inner cytoplasmic leaflet of the plasma membrane to the outer cell surface leaflet. This assay provides an estimation of apoptosis without the changes in cytoplasmic and nuclear volume that might affect the flow cytometric analysis. A significantly higher percentage of apoptotic cells were observed in the splenic leukocyte culture than in the comparable splenic leukocytes cultures exposed to 0.6 mg/l TBT (Fig. 2). Additionally, the percentage of apoptotic cells in pronephric leukocyte cultures increased with increasing TBT concentration (0.3 and 0.6 mg/l TBT). The apparently higher percentage of apoptotic cells in the pronephric leukocyte culture as compared to the splenic cultures exposed to 0.3 mg/l TBT concentration might be explained by differences in the relative proportion of granulocytes and lymphocytes in the cultures. Thus, we sought to identify the relative proportion of cell types and determine whether a particular cell type was sensitive to TBT.
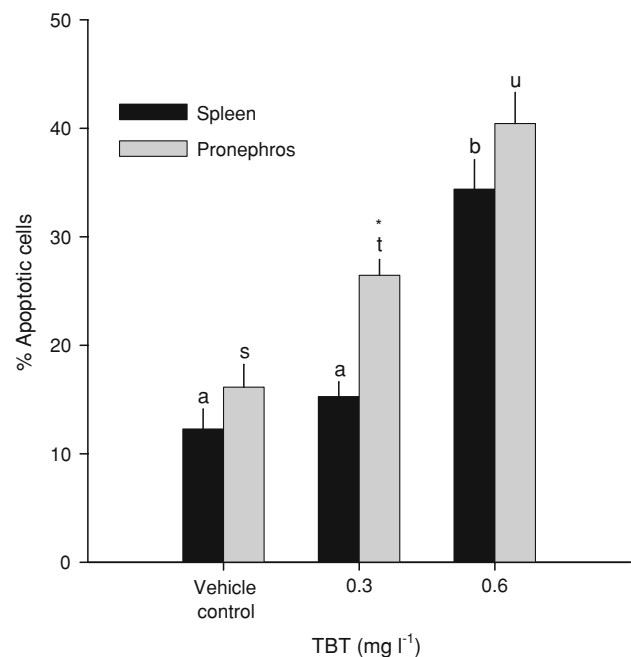

Fig. 1 Mean percent ($\pm$SEM) total viable splenic and pronephric leukocytes following exposure to TBT (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/l) for 24 h ($N = 9$). Percentage of viable leukocytes in the cell culture was measured by the flow cytometry with PI staining

Fig. 2 Mean percent ($\pm$SEM) splenic and pronephric apoptotic leukocytes following exposure to TBT (0, 0.3, and 0.6 mg/l) for 6 h ($N = 9$). Columns with the same superscripts are not significantly different ($p > 0.05$, Kruskal–Wallis test and ANOVA). Significant differences between spleen and pronephros are denoted by an asterisk ($p < 0.05$, Mann–Whitney U-test and Student's t-test)

Two Different Subpopulations of Pronephric Leukocytes

The proportion of viable lymphocytes in the pronephric leukocyte culture decreased significantly with increasing concentrations of TBT after 96 h. The proportion of viable granulocytes in the pronephric leukocyte culture also increased significantly with concentrations of TBT (Fig. 3a–c), suggesting that the lymphocytes in the cultures were more sensitive to TBT. The proportion of viable lymphocytes was significantly higher than that of granulocytes in the control cultures and significantly lower in the presence of 0.1 mg/l of TBT (Fig. 3c). At this point, our experimental results could not distinguish between a decrease in viable lymphocytes or multiplication of granulocytes in culture Therefore, we determined whether the total number of viable cells increased or decreased with TBT treatment. In Fig. 3d, it is clear that the total number of viable cells in the pronephric cell culture decreased significantly with concentrations of

TBT. This observation was verified by microscopic observation (data not shown). In conclusion, salmon pronephric and splenic lymphocytes exhibited a greater sensitivity to TBT.

Mitogenic Response

The functional ability of splenic B-cells to undergo blastogenesis upon LPS stimulation was significantly inhibited in the presence of 0.05, 0.07, or 0.10 mg/l of TBT in the cell cultures (Fig. 4a). In the control cultures, there were no significant differences among treatments in the percentage of blasting B-cells in the cell cultures incubated without LPS (TCM alone). For all treatments, splenic leukocytes treated with LPS had a significantly higher percentage of blasting B-cells than those incubated with TCM alone.

The cell viability was significantly suppressed in the unstimulated cell cultures exposed to 0.10 mg/l of TBT (Fig. 4b). In the LPS stimulated cell cultures, significant
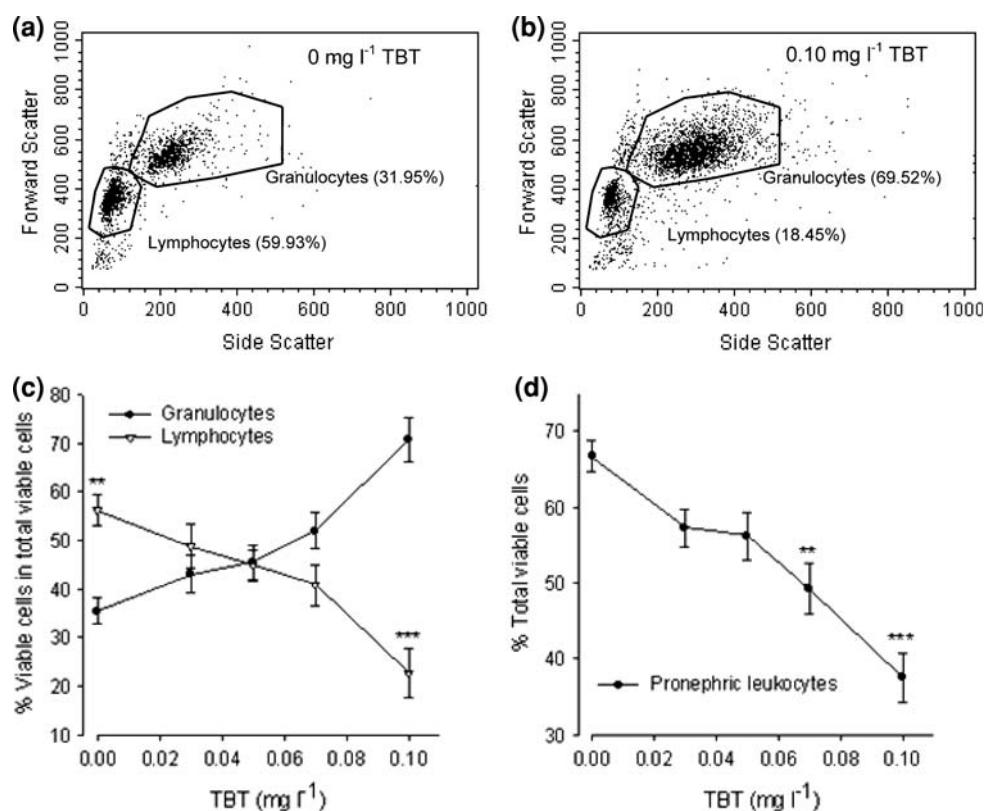

Fig. 3 Effects of exposure of pronephric leukocytes to TBT. Representative flow cytometric forward and side scatter dot plots following incubation for 96 h without TBT (**a**) and with TBT (0.1 mg/l) (**b**). Mean (±SEM) percent viable lymphocytes and granulocytes in the pronephric cell culture exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg/l) for 96 h ($N = 9$) (**c**). Significant differences between lymphocytes and granulocytes are denoted adobe plots of lymphocytes: *** $p < 0.001$, Mann–Whitney U-test and Student's t-test; ** $p < 0.01$; or * $p < 0.05$. Mean (±SEM) percent viable pronephric leukocytes in the cell culture exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg/l) for 96 h ($N = 9$) (**d**). Any TBT treatments that are significantly different from vehicle control (0 mg l^{-1} TBT) are denoted by *** $p < 0.001$, Mann–Whitney U-test and Student's t-test or ** $p < 0.01$
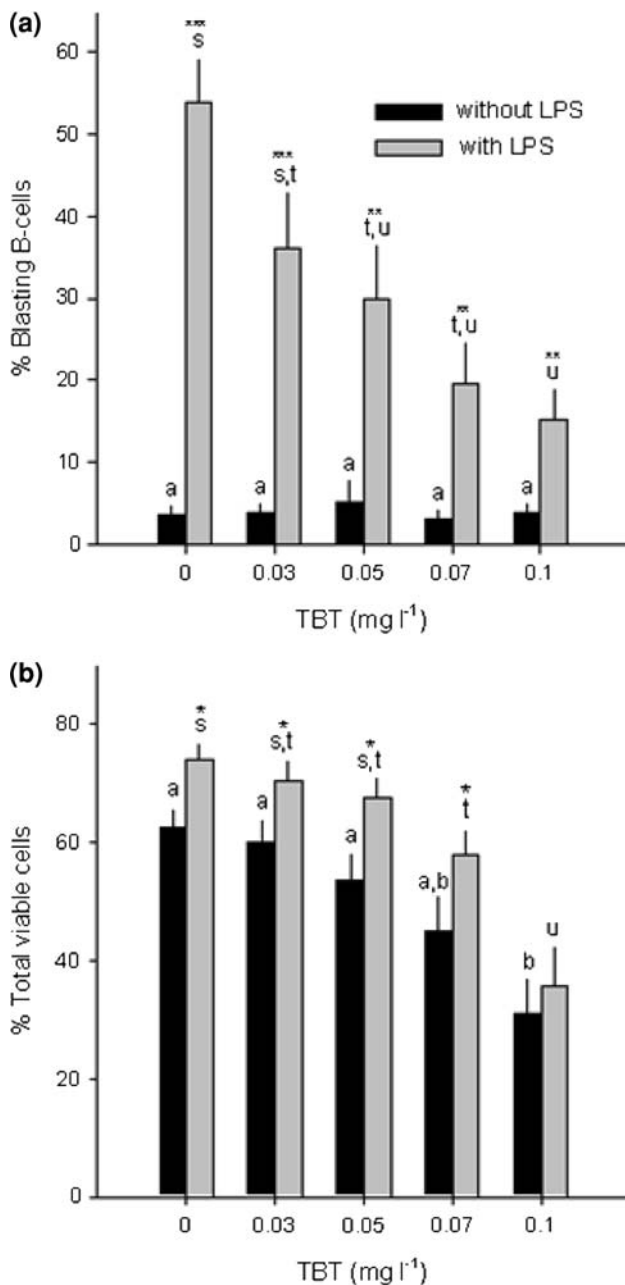
Fig. 4 The effect of in vitro exposures to TBT on blastogenesis of splenic B-cells. Mean (±SEM) percent of blasting splenic B-cells expressing surface Ig (**a**) and percent viable cells (**b**) in the splenic cell culture following exposure to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg/l) with TCM alone or LPS for 96 h (N = 9). Columns that have the same superscripts are not significantly different (p > 0.05, Kruskal–Wallis test and ANOVA). Significant differences between LPS and media are denoted with *** p < 0.001, Mann–Whitney U-test and Student's t-test; ** p < 0.01, or * p < 0.05

Discussion

In our study, incubation of salmon leukocytes in the presence of TBT led to a concentration-dependent decrease in viable cells in cultures. The decrease was attributed to the induction of apoptosis in the treated cells. Apoptosis is a normal and important physiological process for proliferating cells frequently found in the positive and negative selection of thymocytes, morphological development of embryos, or maintenance of the normal tissue (Abelli et al. 1998; Janeway et al. 1999). However, the disorganization of the apoptotic process by TBT could lead to serious health problems and disorders in immunoregulation associated with immunosuppression or immune dysregulation (Alison and Sarraf 1995; O'Halloran et al. 1998); for example, in mammals, macrophages that have ingested apoptotic cells secrete immunosuppressive cytokines such as interleukin (IL)-10, whereas they inhibit secretions of proinflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)α, and IL-12 (Byrne and Reen 2002; Fadok et al. 1998).

lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocytes. This finding is of particular interest because a previous study (Schwaiger et al. 1994) in rainbow trout (*Oncorhynchus mykiss*) demonstrated an elevation in circulating granulocytes following prolonged in vivo exposure to sublethal concentrations of TBT, whereas the number of lymphocytes was significantly reduced. Increase in granulocytes following TBT exposure has also been reported in guppies (Wester and Canton 1987) and rats (Krajnc et al. 1984; Raffray and Cohen 1993). Tada-Oikawa et al. (2008) reported similar results: that murine Th2 lymphocytes were more resistant than Th1 lymphocytes to the toxic effects of TBT. Moreover, they revealed that the higher susceptibility of Th1 lymphocytes was due to lower intracellular glutathione (GSH) levels in Th1 lymphocytes compared to Th2 lymphocytes. Cellular GSH is an antioxidant that that protects the cell from oxidative damages (Meister and Anderson 1983). TBT induces oxidative damage by strong production of reactive oxygen species, and it significantly reduces the cellular content of GSH in rat thymocytes at nanomolar concentrations (Liu et al. 2006; Okada et al. 2000); that is, the susceptibility of leukocytes against TBT might be determined by the intracellular GSH level, although further studies are needed to compare such GSH levels between granulocytes and lymphocytes in fish.

We found that the cell population profiles in cell cultures were different between splenic and pronephric leukocytes. Most splenic leukocytes were lymphocytes (90%), whereas pronephric leukocytes consisted of both lymphocytes (57%) and granulocytes (35%). Because our findings indicate that lymphocytes were more sensitive to

suppression in viability was observed at 0.07 and 0.10 mg/l of TBT. Cell viabilities in LPS-stimulated cell cultures were always higher than those in unstimulated cell cultures, except in the culture exposed to the highest concentration of TBT tested, 0.10 mg/l.

TBT than granulocytes, we hypothesized that splenic leukocyte populations might exhibit a greater sensitivity than pronephric leukocyte populations. However, in this study, there were no statistically significant differences in sensitivity to TBT exposures between splenic and pronephric leukocytes. These findings might relate to the fact that the teleost pronephros is the primary site of hematopoiesis (Yasutake and Wales 1983) and pronephric lymphocytes consist of relatively undifferentiated cells (e.g., stem cell or pro-B-cells) (Milston et al. 2003). Undifferentiated young lymphocytes could be more sensitive to TBT than differentiated, matured cells. The sensitivity of lymphocytes to TBT possibly depends on the level of the cell development. Moreover, the susceptibility to apoptosis induced by TBT could be developmentally regulated in lymphocytes. Antiapoptotic protein, Bcl-2, is a critical mediator of the balance between survival and apoptosis in vertebrates (Inohara and Nunez 2000; Kratz et al. 2006). In mammals, Bcl-2 expression is regulated during lymphocyte development (Lu and Osmond 2000). Immature lymphocytes express low levels of Bcl-2, but this increases at later stages (Gratiot-Deans et al. 1994; Merino et al. 1994). Thus, mature lymphocytes are less sensitive to the induction of apoptosis because they express more Bcl-2 that prevents them from apoptosis (Osborne 1994). As yet, a direct link between dependence of lymphocyte sensitivity to TBT on the level of cell development and the regulation of the Bcl-2 in fish lymphocytes has not yet been reported.

Our data showed that TBT suppressed the cell blastogenesis of surface IgM presenting B-cells. Our findings support O'Halloran et al. (1998), who reported that TBT selectively suppressed the mitogenic activity of LPS-stimulated leukocytes. Following stimulation with LPS (B-cell mitogen), resting B-cells enlarge their size, increase their new RNA and protein synthesis, and develop into B-lymphoblasts (Janeway et al. 1999). The B-lymphoblasts then differentiate into mature plasma cells, which can secrete significant amount of antibodies (Janeway et al. 1999). Therefore, we suggest that when blastogenesis is suppressed by TBT, the amount of circulating antibody can be reduced in fish. We then speculated that the suppression of the humoral immune responses could lead to an increase in disease susceptibility and mortality in fish.

Weyts (1997) concluded that the cortisol-induced suppression of mitogenic responses of carp lymphocytes was due to actual cell death involving apoptosis. Therefore, we investigated cell viability in the cell cultures, which were used for the mitogenic response assay to determine if inhibition of mitogenic responses by TBT was related to actual cell death. Although cell viability was significantly suppressed in the unstimulated cell cultures exposed to 0.1 mg/l of TBT, there were no differences in the percentage of blasting B-cells among all unstimulated cell cultures. Conversely, in the LPS-stimulated cell cultures, the viability was suppressed in the presence of 0.07 and 0.1 mg/l of TBT, and the percentage of blasting B-cells in the viable cells was suppressed at 0.05, 0.07, and 0.1 mg/l of TBT. In fact, in the unstimulated cell cultures, cell death was not related to the percentage of blasting B-cells. In the LPS-stimulated cell cultures, suppression of the mitogenic response in cell cultures exposed to 0.05 mg/l was also not relevant to cell death. This suggests that the cytotoxic effect of TBT on blastogenesis of salmon B-cell could result in an inhibition of mitosis in resting B-cells without cell death. Inhibition of mitogenic responses induced by 0.07 and 0.1 mg/l TBT might be associated with cell death. Misumi (2003) reported that a lower density of viable cells causes lower induction of blasting B-cells in culture following 4 days of incubation with LPS.

The TBT concentrations used in the cell culture were chosen based on results obtained by O'Halloran et al. (1998), Nakata et al. (2002), and Harford et al. (2005). The levels of TBT in seawater sampled from contaminated estuaries have been reported as high as nanogram per liter levels (Lee et al. 2006; Michel et al. 2001). Total butyltin concentrations in fish livers from bays in Washington and Oregon were 290–690 μg/kg wet weight (Krone et al. 1996). The median lethal concentrations (LC_{50}s) of TBT to juvenile Chinook salmon were 54, 20, and 1.5 μg/l after exposures for 6, 12, and 96 h, respectively (Short and Thrower 1986). Compared to the LC_{50} and possible exposure levels encountered by salmon in natural environments, our concentration range between 30 and 100 μg/l appear to be very high. Indeed, Schwaiger et al. (1992) reported that in vivo exposures to 0.6–4.0 μg/l of TBT for 28 days depleted the number of splenic lymphocytes in rainbow trout. However, isolated cells exposed for a short duration seem less sensitive to toxicants than the whole organism exposed for a much longer period of time. In addition, fetal bovine serum in TCM could attenuate TBT cytotoxicity against leukocytes (Umebayashi et al. 2004), which suggested the necessity for such high concentrations of TBT in cell cultures for this study.

In conclusion, in vitro exposure to TBT induced the immunosuppression in splenic and pronephric leukocytes isolated from Chinook salmon and significantly reduced cell viability following the induction of apoptosis. Interestingly, pronephric granulocytes were more resistant than pronephric lymphocytes to the toxic effects of TBT. Furthermore, splenic leukocytes cultured with LPS in the presence of TBT demonstrated a concentration-dependent inhibitory effect on the ability of mitogen-stimulated B-cells to undergo blastogenesis. Those findings suggest that adverse effects of TBT on function and development of fish immune systems could lead to an increase in disease susceptibility and its subsequent ecological implications.

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