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Original Article

Tributyltin (TBT) Increases TNFα mRNA Expression and Induces Apoptosis in the Murine Macrophage Cell Line in Vitro

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Abstract

Objective: Tributyltin (TBT) compounds have been widely used as antifouling agents for ship-bottom paint. The immune system is a target of TBT intoxication. We evaluated the effects of TBT chloride in macrophages, which have critical roles in the immune system, using a murine macrophage lineage cell line, J774.1, *in vitro*.

Methods: We examined tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and *c-jun* mRNA expression in J774.1 cells. The effects of TBT on the apoptosis of J774.1 cells were examined by determining the percentage of TUNEL-positive cells and caspase-3 activity.

Results: The mean values of the viabilities of J774.1 cells exposed to TBT decreased dose-dependently. The relative mRNA expression of TNFα increased dose-dependently, however, that of IL-1β was not significantly different among the groups. The mean percentage of TUNEL-positive cells increased dose-dependently. Increases in the caspase-3 activities of J774.1 cells were observed in the groups exposed to higher concentrations of TBT. The mean value of relative mRNA expression of c-Jun transcription factor increased dose-dependently.

Conclusions: The increases in the percentage of TUNEL-positive cells and in caspase-3 activity suggested that exposure to TBT induces apoptosis of J774.1 cells. The increases in the mRNA expression of TNFα and *c-jun* by TBT may be related to apoptosis in macrophages.

Key words: tributyltin, immunotoxicity, macrophage, apoptosis

Introduction

Organotin compounds have been widely used as biocides, fungicides and antifouling agents for ship-bottom paint (1, 2). In particular, tributyltin (TBT) compounds have been heavily used as antifouling agents, and pollution by TBT in the marine environment has become a serious problem worldwide. Because of the possible health hazards of TBT, the production, import and use of TBT oxide were prohibited by law in 1990 in Japan (3). Also, the import and use of thirteen other kinds of TBT compounds require registration with the Ministry of Economy, Trade and Industry in advance. In accordance with administrative guidance, Japanese manufacturers decided to stop the production of ship-bottom paint containing TBT compounds in 1997 as a self-imposed control. However, TBT compounds are still used as ship-bottom paint in other countries. The pollution of water by TBT compounds is still being reported worldwide and in Japan (4–6). Because of water pollution, TBT compounds have been detected in fish and shellfish, which are frequently eaten by humans (4, 7). TBT compounds are detected not only in fish and shellfish but also in their processed products (8). Even if cooked, the organotin compounds in fish and shellfish are not destroyed (9). There is a risk of being exposed to TBT compounds by eating fish and shellfish, especially in Japan, as the Japanese eat relatively large amounts of fish. TBT compounds have been detected in human blood (10) and in the liver (11).

The toxic effects of TBT compounds have been studied. The immune system is a target of TBT intoxication (12, 13). Studies of the immunotoxicity of TBT have mostly focused on

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the toxicity for lymphocytes. Low levels of TBT induced cell death of lymphocytes. TBT has induced apoptosis and caspase activation in human leukemia T cells (14, 15), peripheral T lymphocytes (16) and rat thymocytes (17, 18). However, little is known about the immunotoxicity of TBT compounds for macrophages. Macrophages have a variety of functions, such as producing cytokines (interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNFα)), phagocytosis and antigen-presenting. Among the cytokines produced by macrophages, TNFα induces apoptosis of cells, and is related to c-Jun transcription factor (19).

There are many problems among the effects of TBT on macrophages that need to be studied. These problems include the effects of TBT on the macrophage production of cytokines, which are an index of the functions of macrophages, the level of TBT that induces cell death of macrophages, and the relation of apoptosis to cell death induced by TBT. Investigation of these problems is worthwhile for the elucidation of the mechanism of toxic effects on macrophages by TBT.

The mRNA expressions of TNFα and IL-1β in macrophages, evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR), have previously been used as indexes of immunotoxicity (20, 21). We evaluated the effects of TBT chloride on TNFα and IL-1β mRNA expression in a murine macrophage lineage cell line, J774.1, *in vitro*. In addition, we examined the effects of TBT on the apoptosis of J774.1 cells by determining the percentage of TUNEL-positive cells, and caspase-3 activity. The effect of TBT on *c-jun* mRNA expression was also evaluated.

Materials and Methods

J774.1 cells

J774.1 cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai). J774.1 cells were cultured in RPMI 1640 with penicillin G at 100 U/ml and streptomycin at 100 μg/ml (Nikken, Kyoto) with 5% [v/v] heat-inactivated fetal bovine serum (FBS, Equitec-Bio, Kerrville, TX).

The exposure of J774.1 cells to TBT

J774.1 cells were cultured and activated by LPS following the protocol used in the previous study (22). J774.1 cells were aliquoted in a 24-well culture plate at 1×10^6 cells/well. TBT chloride (Tokyo Kasei, Tokyo) was added to the wells at concentrations of 0, 0.5, 1.0 and 1.5 μM. TBT chloride was dissolved in ethanol and diluted by the culture medium. The final concentration of ethanol in each well was 0.1%. The number of wells for each concentration was 6. The plate was incubated in a humidified incubator at 37°C with 5% CO_2 for 18 hours. After 18 hours, LPS was added to each well. The final concentration of LPS was 100 nM. The cells were incubated under the same conditions for an additional 6 hours.

Measurement of cell viability and mRNA expression for cytokines

Cell viability was determined using the trypan blue exclusion method. Total RNA was extracted from the J774.1 cells at 24 h following exposure to TBT using TRIzol reagent (Life Technologies, Frederick, MD) according to the manufacturer's protocol. The cDNA was synthesized from mRNA using a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ). RT-PCR was used to analyze the mRNA expression for TNFα and IL-1β in J774.1 cells, and for β-actin (internal control), as described previously (20). The sense and anti-sense primers used were 5'-GTTCTATGGCCCAGACCCTCACA-3' and 5'-TCCCAGGTATATGGGTTCATACC-3' for TNFα (23), 5'-GCAACTGTTCCTGAACTCA-3' and 5'-CTCGGAGCCT-GTAGTGCAG-3' for IL-1β (24), and 5'-ATGGATGAC-GATATCGCT-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' for β-actin. The thermal cycles consisted of denaturation at 94°C for 30 s, annealing (TNFα, 54°C; IL-1β, 56°C and β-actin, 48°C) for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The number of cycles optimized for each primer was 35. The amplification products were fractionated on 2% agarose (Life Technologies) gel containing 0.476 μM ethidium bromide, and analyzed and quantified using Print Graph Imagesaver and Lane & Spot Analyzer Ver 5.0 (ATTO, Tokyo). The quantified value for each cytokine was adjusted by the respective β-actin band. The mean values of relative expression of each cytokine were compared among the groups.

TUNEL assay

The induction of apoptosis in J774.1 cells by TBT chloride was examined. To detect apoptotic cells, the sensitive TUNEL method (25, 26) was used. An apoptosis in situ detection kit (Wako, Osaka) was used for the TUNEL method, following the manufacturer's protocol.

Measurement of caspase-3 activity

The caspase-3 activity in J774.1 cells exposed to TBT chloride was measured. The CaspACE™ Assay System Colorimetric (Promega Corp. WI) was used for the measurement of caspase-3 activity. The measurements were made following the manufacturer's protocol. Briefly, J774.1 cells were exposed to TBT chloride and harvested. The harvested J774.1 cells were centrifuged at 450×g for 10 minutes at 4°C. The cells were washed with ice-cold phosphate buffered saline (PBS) and resuspended in a Cell Lysis Buffer. The cells were lysed by freezing and thawing, then incubated on ice for 15 minutes. Freeze-thaw cycles were repeated twice. The cell lysates were centrifuged at 15,000×g for 20 minutes at 4°C. The supernatant of each cell lysate was used as the cell extract. The caspase assay was performed in a 96-well plate. The following materials were added to each well: 32 μl of caspase assay buffer, 2 μl of dimethyl sulfoxide (DMSO) and 20 μl of 100 mM of dithiothreitol (DTT). The cell extract from each sample was added to each respective well at a volume of 20 μl. The sample volume of each well was adjusted to 98 μl by distilled water. Finally, 2 μl of 10 mM DEVD p-nitroaniline (pNA) substrate was added to each well. The plate was covered with plate sealer and incubated at 37°C for 4 hours. The absorbance in the wells was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, CA). Caspase-specific activities were calculated using the standard curve of pNA. Each caspase activity was adjusted by each protein concentration of the cell extract determined by

the Bradford method (27) using a 7010 Clinical Spectrophotometer (Hitachi, Tokyo).

Measurement of mRNA expression of c-jun

The expression of the *c-jun* gene was examined by the mRNA analysis used for RT-PCR. The sense and anti-sense primers used for *c-jun* were 5'-ATGCCCTCAACGCCTCGTTCCTCC-3' and 5'-CTGCTCGTCGGTCACGTTCTTGGG-3' (28). The thermal cycles consisted of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The number of cycles optimized for *c-jun* was 40.

Statistical Analysis

The data of the control and exposure groups were compared by one-way ANOVA followed by Fisher's PLSD test using Statview version 5.0 (SAS Institute, Cary, NC).

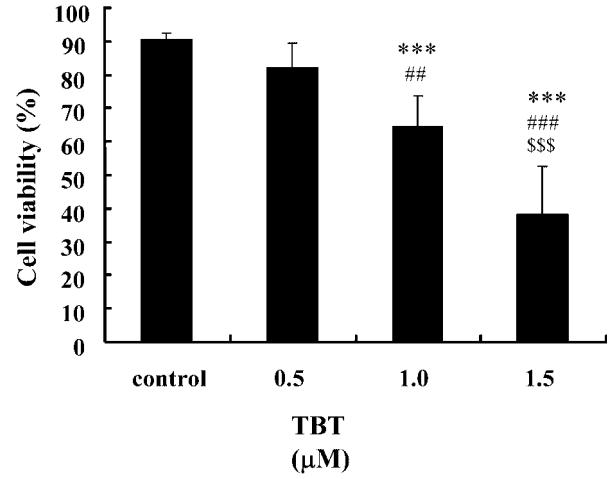

Fig. 1 The cell viability of J774.1 cells exposed to TBT chloride. Cell viability was determined using the trypan blue exclusion test at 24 h following exposure to TBT chloride. Each bar represents mean value, and error bars represent standard error (n=6). ***; p<0.001 compared to the control. ##; p<0.01, ###; p<0.001 compared to the 0.5 μM group. $$$; p<0.001 compared to the 1.0 μM group.

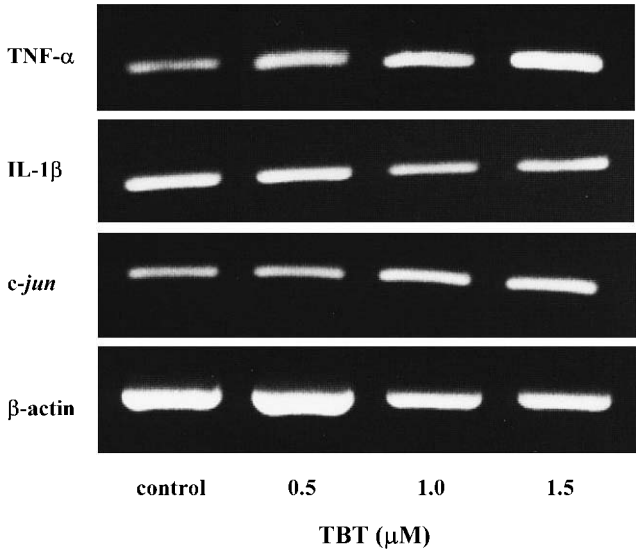

Fig. 2 Polymerase chain reaction (PCR) amplified products of tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), *c-jun* and β-actin in J774.1 cells.

Results

The mean values of the viability of J774.1 cells exposed to TBT were 90.3%, 81.9%, 64.6% and 38.1% for the control and the 0.5 μM, 1.0 μM and 1.5 μM exposure groups, respectively (Fig. 1). The cell viabilities decreased dose-dependently.

Figure 2 presents typical examples of the bands of amplified product for TNFα, IL-1β, *c-jun* and β-actin in the J774.1 cells of different treatment groups. The relative mRNA expressions of TNFα were increased dose-dependently from the control group to the 1.5 μM exposure group (Fig. 3). Figure 4 demonstrates the effects of TBT on the relative expression of IL-1β of J774.1 cells. The relative mRNA expressions of IL-1β were not significantly different among the groups.

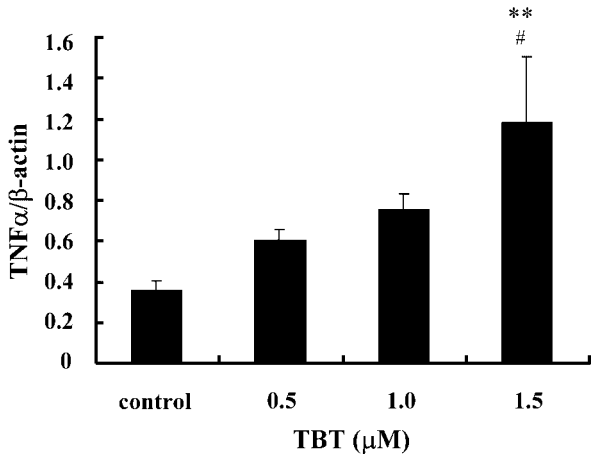

Fig. 3 The relative mRNA expression of TNFα in J774.1 cells exposed to TBT chloride. RNA was extracted from the J774.1 cells at 24 h following exposure to TBT chloride. RT-PCR was used to analyze the mRNA expression for TNFα and β-actin in J774.1 cells. Each bar represents mean value, and error bars represent standard error (n=6). **; p<0.01 compared to the control. #; p<0.05 compared to the 0.5 μM group.

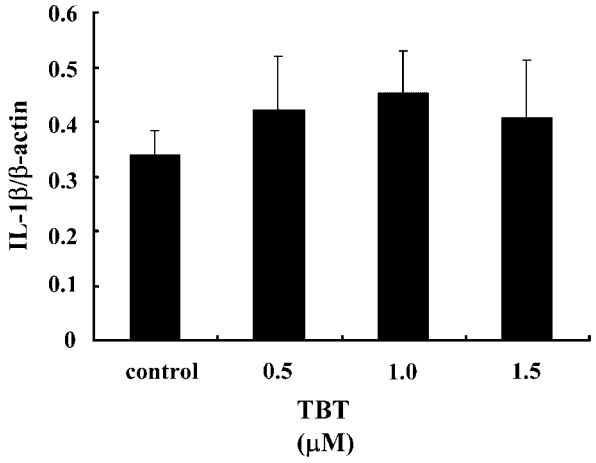

Fig. 4 The relative mRNA expression of IL-1β in J774.1 cells exposed to TBT chloride. RNA was extracted from the J774.1 cells at 24 h following exposure to TBT chloride. RT-PCR was used to analyze the mRNA expression for IL-1β and β-actin in J774.1 cells. Each bar represents mean value, and error bars represent standard error (n=6).

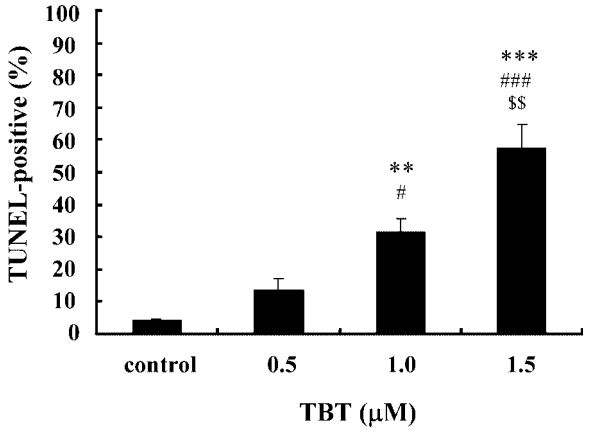

Fig. 5 The percentages of TUNEL-positive cells in J774.1 cells exposed to TBT chloride. TUNEL-positive cells were detected using an apoptosis in situ detection kit (Wako, Osaka). Each bar represents mean value, and error bars represent standard error (n=6). **; p<0.01, ***; p<0.001 compared to the control. #; p<0.05, ###; p<0.001 compared to the 0.5 μM group. $$; p<0.01 compared to the 1.0 μM group.

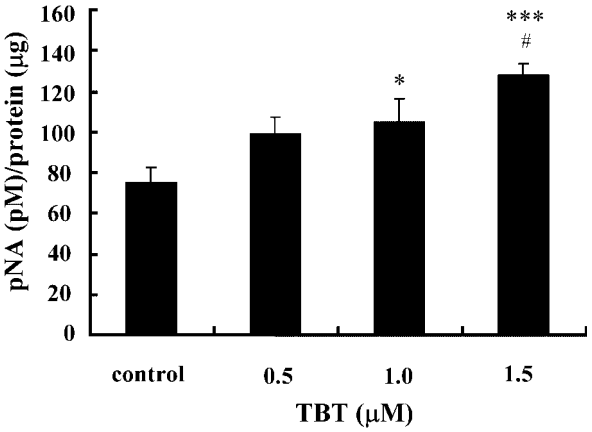

Fig. 6 The caspase-3 activity in J774.1 cells exposed to TBT chloride. J774.1 cells were exposed to TBT chloride and harvested. The caspase-3 activity in the supernatant of the cell lysate was determined using DEVD p-nitroaniline substrate. The absorbance was measured at 405 nm. Each bar represents mean value, and error bars represent standard error (n=6). *; p<0.05, ***; p<0.001 compared to the control. #; p<0.05 compared to the 0.5 μM group.

The mean values of the percentage of TUNEL-positive cells were 4.1%, 13.5%, 31.3% and 57.2% for the control and the 0.5 μM, 1.0 μM and 1.5 μM exposure groups, respectively (Fig. 5). The percentage of TUNEL-positive cells increased dose-dependently. Figure 6 illustrates the caspase-3 activities in J774.1 cells exposed to TBT. A modest increase in the caspase-3 activities in J774.1 cells was observed in the groups exposed to higher concentrations of TBT.

The mean value of relative mRNA expression of c-Jun transcription factor increased dose-dependently (Fig. 7).

Discussion

TBT induces thymic atrophy and immunosuppression (12, 13). Much is known about TBT toxicity for lymphcytes, however, little is known about TBT toxicity for macrophages. Therefore, for the elucidation of the mechanism of immunotoxicity of TBT, study focused on macrophages is essential. We examined the immunotoxicity of TBT for macrophages, and used J774.1 cells, due to their wide use as *in vitro* models of macrophages.

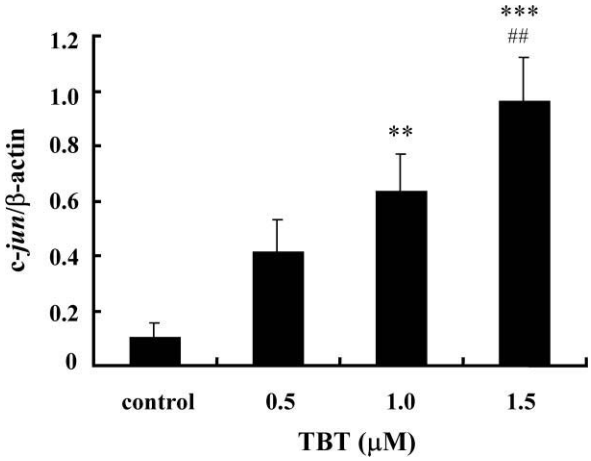

Fig. 7 The relative mRNA expression of *c-jun* in J774.1 cells exposed to TBT chloride. RNA was extracted from the J774.1 cells at 24 h following exposure to TBT chloride. RT-PCR was used to analyze the mRNA expression of *c-jun* and β-actin in J774.1 cells. Each bar represents mean value, and error bars represent standard error (n=6). **; p<0.01, ***; p<0.001 compared to the control. ##; p<0.01 compared to the 0.5 μM group.

TNFα and IL-1β are major cytokines produced by activated macrophages (29). These cytokines are related to the functions of macrophages. In addition, apoptosis, one of the mechanisms of cell death, is closely related to TNFα (30). TBT induces apoptosis in several kinds of cells (13–18). We hypothesized that TBT induces apoptosis in macrophages, and that the mechanism of the apoptosis induced by TBT is related to TNFα or *c-jun*. We used the mRNA expression of TNFα, IL-1β and *c-jun* as indexes of toxicity for macrophages in the current study.

The cell viability of J774.1 cells by TBT exposure decreased dose-dependently. Yu et al. (31) demonstrated that the cell viability of CCRF-CEM human T lymphoblastoid cells exposed to 1 μM TBT was 29% of that of control cells after 24 h. Incubation with 5 μM of TBT oxide caused a rapid decrease in rat thymocyte viability (13). After 6 h of exposure, the cell viability was under 30%. Also, incubation with 1 μM TBT oxide caused a slight reduction of cell viability. In the current study, the mean cell viability of the 1.5 μM group was 38.1% and that of the 1.0 μM group was 64.6%. At levels of 1.0 μM and over, significant decreases in cell viability were observed. The levels of TBT that caused macrophage cell death were similar to the levels at which lymphocyte cell death was induced in previous studies.

The relative mRNA expressions of TNFα in J774.1 cells were increased by TBT exposure dose-dependently. The relative mRNA expression of IL-1β in J774.1 cells was not affected by TBT exposure. It is suggested that TBT selectively increases TNFα gene expression in macrophages, among the cytokines. In an *in vivo* study, Corsini et al. (32) demonstrated that in the ears of BALB/c mice painted with different amounts of TBT (67–536 nM in acetone), the induction of TNFα increased dose-dependently 2 h and 4 h after TBT treatment. The induction of TNFα gene expression by TBT should be studied further for

alternate routes of administration *in vivo*.

The increase in TNFα mRNA gene expression may be related to the apoptosis of macrophages induced by TBT *in vitro*. We used the TUNEL method as a simple and sensitive technique for detecting apoptotic cells. The percentages of TUNEL-positive J774.1 cells increased dose-dependently, from 4.1% in the control to 57.2% in the 1.5 μM exposure group. The activation of caspases has a critical role in apoptosis (33). In particular, activation of caspase-3 facilitates the progression of cells into the final stage of apoptosis. In the current study, an increase in caspase-3 activities in J774.1 cells was observed in the groups exposed to higher concentrations of TBT. The increases in the percentage of TUNEL-positive cells and in caspase-3 activity strongly suggested that exposure to TBT at the level of 1 μM and over induces apoptosis in J774.1 cells. Stridh et al. (15) examined the induction of apoptosis in the human Hut-78 T cell line after exposure to TBT chloride. Hut-78 apoptotic cells appeared at 70% after exposure to 2 μM TBT for 3 hours. Lavastre and Girard (34) demonstrated that apoptosis in human neutrophil was caused by 1 μM TBT chloride exposure after 24 h. Zucker et al. (35) demonstrated that apoptosis of rat thymocytes was induced by TBT exposure at concentrations of 1.0 μM and over. The TBT levels causing macrophage apoptosis in the current study were similar to those causing apoptosis in other immune cells in previous studies. However, the extent of the increase in caspase-3 activity by TBT was smaller than that in TUNEL-positive cells. It is suggested that necrosis, an other mechanism of cell death, also plays a role in the macrophage cell death induced by TBT.

The expression of *c-jun* was induced in response to treatments that frequently trigger apoptosis by TNFα (19). Transcriptional c-Jun factor plays an active role in apoptosis via a transcriptional mechanism in the JNK pathway in a variety of cells (36). Bossy-Wetzel et al. (37) demonstrated that the overexpression of *c-jun* mRNA by β-estradiol induced apoptosis in NIH 3T3 fibroblasts. Zhou et al. (38) demonstrated that the overexpression of *c-jun* mRNA induced by cadmium in both the testes and the ventral prostate occurred similtaneously with the peak time of apoptosis in these two tissues. In the current study, the mean value of the relative mRNA expression of c-Jun transcription factor was increased by TBT dose-dependently. The increase in *c-jun* mRNA expression may be related to the apoptosis of macrophages induced by TBT.

In conclusion, the TBT levels that caused the cell death of macrophages were similar to those causing the cell death of lymphocytes, which are known as target cells of TBT. Increases in the mRNA expression of TNFα and *c-jun* were induced by TBT. The increases in the percentage of TUNEL-positive cells and caspase-3 activity in the high TBT exposure groups suggest the induction of apoptosis by TBT. TNFα and *c-jun* may be related to the apoptosis of macrophages induced by TBT. The effects of TBT on macrophages should be studied *in vivo*.

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