Bull Environ Contam Toxicol (2012) 89:704–711
DOI 10.1007/s00128-012-0760-2

Stress-Response Protein Expression and DAF-16 Translocation were Induced in Tributyltin-Exposed *Caenorhabditis elegans*

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Received: 5 February 2012 / Accepted: 20 July 2012 / Published online: 9 August 2012

Abstract Exposure to tributyltin (TBT) with graded sublethal doses (0, 1, 10, 50 and 200 nM) resulted in the release of reactive oxygen species (ROS) and DNA damage in the nematode *Caenorhabditis elegans.* After the worms carrying transgenic reporters were exposed to TBT, the expressions of superoxide dismutase (SOD-3), glutathione S-transferase (GST-4) and heat shock proteins (HSP-4, HSP-16.2 and HSP-70) were semi-quantified after exposure. The results indicated that TBT caused dose-dependent induction of SOD-3, GST-4, HSP-4 and HSP-70. Furthermore, TBT exposure also induced DAF-16 translocation from cytoplasm to nucleus. The results implicated that *C. elegans* might be a potential animal model for TBT level monitoring and toxicity assessment.

Keywords Stress-response protein · DAF-16 translocation · Tributyltin · In vivo · *Caenorhabditis elegans*

Tributyltin (TBT), a representative organotin compound, is used in wood preservatives, agricultural pesticides, and antifouling paints on ship hulls or fishnets due to its biocidal properties. Although the use of TBT in antifouling paints was restricted or banned in most countries (Champ 2000; van Wezel and van Vlaardingen 2004), TBT and its metabolites were still detectable in many regions (Berto et al. 2007; Viglino et al. 2004). World Health Organization proposed a provisional guideline value of 3.36 nM for tributyltin oxide (TBTO) in drinking-water (WTO 1996). However, it was reported that butyltins, including TBT, were even detected in human blood at concentrations ranging between 50 and 400 nM (Whalen et al. 1999). Previous results also showed that contamination with TBT in the aquatic environment of China was very serious (Jiang et al. 2001; Yang et al. 2006). Experimental data showed the widespread occurrence of butyltin compounds in the Chinese aquatic environment, and in some locations, the concentrations of TBT are higher than the acute and chronic toxicity threshold of sensitive fresh water or marine organisms (Jiang et al. 2001).

Persistent organic pollutants (POPs) are organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes. Because of this, they have been observed to persist in the environment, to be capable of long-range transport, bioaccumulate in human and animal tissue, biomagnify in food chains, and to have potential significant impacts on human health and the environment. Tributyltin compounds are moderately to highly POPs that bioconcentrate up the marine predators' food chain. Exposure to TBT resulted in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks and fish species (Gibbs and Bryan 1986; McAllister and Kime 2003). TBT compounds were also highly hepatotoxic, neurotoxic and/or immunotoxic to animals (Fent 1996; Hoch 2001; Appel 2004). Furthermore, it was demonstrated that the apoptotic pathway followed by TBT started with an increase of intracellular Ca^{2+} level, then continued with release of reactive oxygen species (ROS) and cytochrome c from mitochondria, activation of caspases, and finally resulted in DNA fragmentation (Gennari et al. 2000). Due to the high risks presented to human and animals health, TBT level monitoring and toxicity assessment have received many concerns in the last decade.

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Many techniques have been developed to assess the risks of TBT to the environment. However, TBT induced oxidative stress to the intact animal in vivo has not been well documented due to the lack of adequate animal models. In 1990, Williams and Dusenbery proposed that the nematode *Caenorhabditis elegans* can be used as animal models to monitor the toxic effects of heavy metals (Williams and Dusenbery 1990). Since then, *C. elegans* has been widely used as an in vivo animal model for risk assessment of many environmental toxicants using multiple sublethal endpoints. In the present investigation, the ROS generation and DNA damage were confirmed in the nematode after TBT exposure. Then transgenic *C. elegans* strains carrying green fluorescent protein (GFP) fusion reporters were exposed to TBT and several antioxidant proteins were quantified. The main objectives of the present study are to provide the evidences of TBT toxicity and to know how the stress-response proteins expression and DAF-16 translocation were regulated by TBT in *C. elegans* with a rapid method in vivo. Finally, we aimed to gain a potential way to monitor TBT pollution in the environment.

Materials and Methods

Worm strains used in the present study were the wild type N2, WS1433, CF1553, CL2166, SJ4005, BC10060, CL2070, TJ375 and GR1352, all of which were provided by the *Caenorhabditis* Genetics Center funded by the National Center for Research Resources of National Institutes of Health (NIH). WS1433 was constructed with low-copy integrated array of 1144 bp *hus-1* promoter and genomic coding sequence fused to GFP, and other transgenic strains were constructed with the corresponding promoter regions tagged with GFP. Worms were cultured at 20°C in Petri dishes on nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 as food. Synchronized worm cultures were obtained by lysing gravid hermaphrodites in an alkaline hypochlorite solution as described previously (Sulston and Hodgkin 1988). For the strain BC10060, only wild-type phenotype worms which expressed HSP-70::GFP were maintained and picked for experiments. Tributyltin chloride (96 % purity) was a commercial product of Sigma-Aldrich (United States).

The procedures for animal handling and chemical exposure were conducted as described previously (Williams and Dusenbery 1990). Briefly, TBT was dissolved in dimethyl sulfoxide (DMSO) and diluted to final concentrations of 0, 1, 10, 50, 200 nM in K-medium (52 mM NaCl and 32 mM KCl in ddH_2O), *E. coli* strain OP50 was added to the medium as a food source. Aliquots of 1.0 mL test solution were dispensed into a Costar 12-well tissue plate. For animal exposure, synchronized young adult hermaphrodites were picked and transferred into the wells except for the DAF-16 translocation assay. Worms were grown at 20°C and removed for further analysis. Four replicates were conducted for each assay to ensure that all the results were acceptable. All experiments in this study were conducted in accordance with national and institutional guidelines for the protection of animal welfare.

For ROS determination, 25 synchronized L4-staged N2 hermaphrodites were treated with 0, 1, 10, 50, and 200 nM TBT for 24 h. Worms were piped and added to 150 µL PBS containing 0.1 % Tween 20 and then homogenized with glass homogenizer followed by sonication on ice. Aliquots of 100 µL samples were added to 96-well plates after vortex. Aliquots of 10 µL 100 µm CM-H2DCFDA in PBST were added to the samples and incubated at 37°C for 45 min in a fluorescent microplate reader (Molecule Device SpectraMax M2e). The relative fluorescence units (RFU) per 25 worms were determined at excitation 485 nM and emission 540 nM wavelength.

For DNA damage assay, synchronized WS1433 young adult hermaphrodites were treated with indicated TBT concentrations for 24 h. HUS-1::GFP foci formation was quantified by counting the number of bright foci present in pachytene germ cells according to Parusel et al. (2005). Fluorescent images were captured with a ZEISS Axio Imager microscope equipped with an AxioCam digital CCD camera.

To determine the effects of TBT on the levels of superoxide dismutase (SOD-3), glutathione S-transferase (GST-4) and heat shock proteins (HSP-4, HSP-16.2 and HSP-70) in *C. elegans*, synchronized transgenic worm strains were transferred to 1 mL of K-medium with control or TBT solutions at indicated times. Animals were picked and mounted on 2 % agar pads in 60 µg/mL levamisole and examined with an Olympus IX71 fluorescence microscope equipped with FITC excitation filter. Pictures were gotten with an Olympus DP72 camera. The relative fluorescence intensities were semi-quantified using the Adobe Photoshop software.

The GR1352 strain was used to examine the intracellular distribution of DAF-16 in the living nematode. In this strain, the gene coding for GFP was fused to the *daf-16* gene (Lee et al. 2001). To determine whether TBT exposure affected the DAF-16 translocation from cytoplasm to nucleus, adult hermaphrodites were lysed with standard protocols and hatched in M9 buffer [3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL $MgSO_4$ (1 M), 1 L ddH_2O] for 18 h. L1 larva were inoculated to NGM until early L2, worms were washed and transferred to K-medium containing graded doses of TBT for 24 h. Worms were transferred to 2 % agar pads in 60 µg/mL levamisole and observed under an Olympus IX71 fluorescence microscope.

The degree of nuclear translocation of DAF-16 was evaluated by counting the number of worms showing an either weak or strong nuclear GFP fluorescence and computing the relative share (%) of these worms in relation to the total number of worms observed. An image was taken of each worm, and only intestinal cells were analyzed for DAF-16 translocation.

All values were expressed as mean ± standard error (SE). Significant differences ($p < 0.05$) between different treatments or different time points were tested using analysis of variance (ANOVA) followed by Duncan's multiple comparison test.

Results and Discussion

Prior to the main experiments, solvent control experiments were conducted to compare the effect of the non-solvent control group and the solvent control group, which showed that 0.5 % DMSO did not provoke any significant effect in all the tests (data not shown). The controls presented in the context of this paper were solvent controls.

To determine whether TBT exposure would induce the excessive generation of ROS, synchronized N2 young adult hermaphrodites were exposed to graded doses of TBT for 24 h in K-medium, and the RFU was quantified after CM-H2DCFDA staining. As shown in Fig. 1, TBT exposure significantly increased the release of ROS in *C. elegans*. The RFU per 25 worms increased from 90.62 ± 5.87 at untreated control to 117.87 ± 4.56 after exposing to 1 nM of TBT for 24 h ($p < 0.05$). With doses increase, RFU increased significantly. These results suggested that TBT-induced ROS release was in a dose-dependent manner.

The checkpoint gene *hus-1* is known to be required for DNA damage-induced cell cycle arrest and apoptosis.

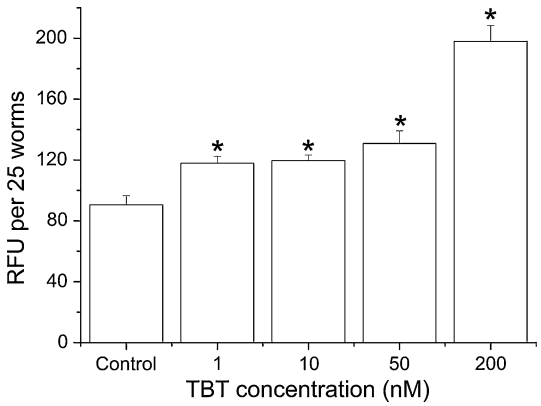

Fig. 1 TBT exposure caused dose-dependent increase in ROS release in the worm after TBT exposure for 24 h. All values are represented as mean ± SE, n = 25. *Error bar* with *asterisk* represents statistical significance ($p < 0.05$) compared to that of the control

HUS-1::GFP localizes diffusely in proliferating germ nuclei under normal conditions. In response to DNA damage, HUS-1::GFP relocalizes in the nucleus and concentrates at distinct nuclear foci that overlap with chromatin believed to be sites of DNA breaks (Hofmann et al. 2002). To test whether TBT-induced DNA damage could be detected by the checkpoint machinery, young adult hermaphrodites of HUS-1::GFP strain WS1433 were treated with TBT for 24 h. The present study showed that exposure to 50 and 200 nM TBT significantly induced the formation of nuclear HUS-1::GFP foci in the germline comparing to that of control ($p < 0.05$, Fig. 2).

In the present study, *C. elegans* strain muIs84 [pAD76(*sod-3*::GFP)] (CF1553) carrying GFP reporter gene was exposed to graded concentrations of TBT for 12 h and 24 h. Green expression in head was measured in this assay. As indicated in Fig. 3, SOD-3 was induced at higher concentration of TBT exposure ($p < 0.05$) after 12 or 24 h exposure, however, SOD-3 expression exhibited decline tendency by TBT exposure from 10 to 50 nM.

To test the roles of GST-4 by TBT exposure in *C. elegans*, the transgenic worm strain CL2166 carrying mammalian GST homolog were exposed to graded doses of TBT for 12 and 24 h. GST-4::GFP expressed in the anterior of the body, mainly in the pharynx region and intestine. TBT exposure induced significant increase of GST-4 in the pharynx region and intestine with the dosage increase (Fig. 4). The relative fluorescence unit increased 2.13 and 2.92 folds at the dose of 200 nM TBT for 12 and 24 h exposure.

To test whether TBT exposure will cause an increase in heat shock protein expression, the transgenic worm strains carrying *hsp-4*::GFP (SJ4005), *hsp-70*::GFP (BC10060) and *hsp-16.2*::GFP (CL2070 and TJ375) were exposed to 0, 1, 10, 50, 200 nM TBT for 24 h. In *C. elegans*, HSP-4 has a constitutive expression at the posterior part of the intestine. Under 50, 200 nM of TBT exposure, HSP-4 expression increased significantly (Fig. 5c). The results indicate that high doses of TBT could induce the expressions of small molecullar HSPs. HSP-70 mainly expressed at the posterior of the body. As shown in Fig. 3, TBT exposure significantly increased the expression of HSP-70. An *hsp-16.2* reporter fusion, expressed broadly but most strongly in muscle and hypodermis, is induced by heat shock or other environmental stresses. However, no significant alterations of HSP-16.2 expression were observed at all doses of TBT exposure ($p > 0.05$, data not shown) in the present research.

The *C. elegans* DAF-16/FOXO is a transcription factor that regulates multiple gene transcription by cytoplasm to nucleus translocation. To test whether TBT exposure would induce the translocation of DAF-16, we tested DAF-16 translocation in L2-L3 larval animals. As indicated in Fig. 6 and Table 1, TBT induced DAF-16 from cytoplasm

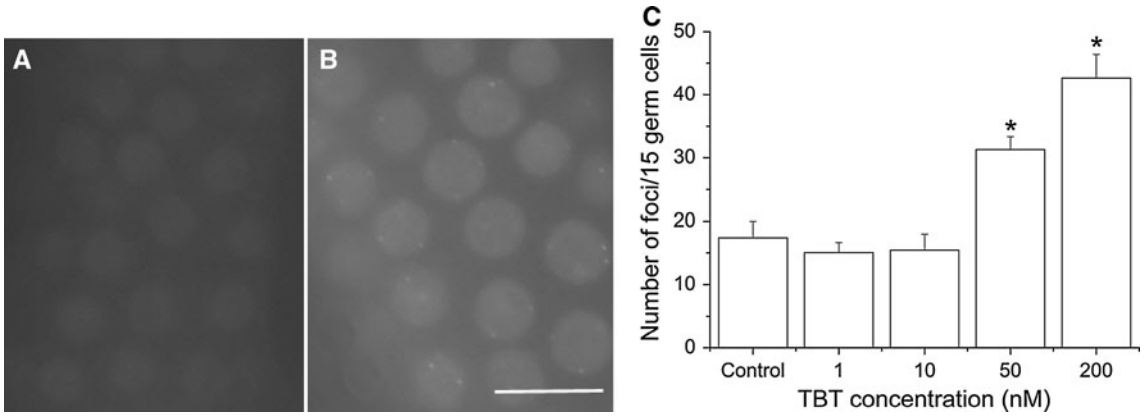

Fig. 2 Formation of nuclear HUS-1::GFP foci in the pachytene germline was induced by TBT exposure. **a** Control and **b** representative microphotographs of HUS-1::GFP foci in the pachytene germline with 200 nM of TBT. *Bar* represents 5 μm. **c** Quantification of nuclear HUS-1::GFP foci induced by TBT exposure. All values are represented as mean ± SE, n = 20. *Error bar* with *asterisk* represents statistical significance ($p < 0.05$) compared to that of the control

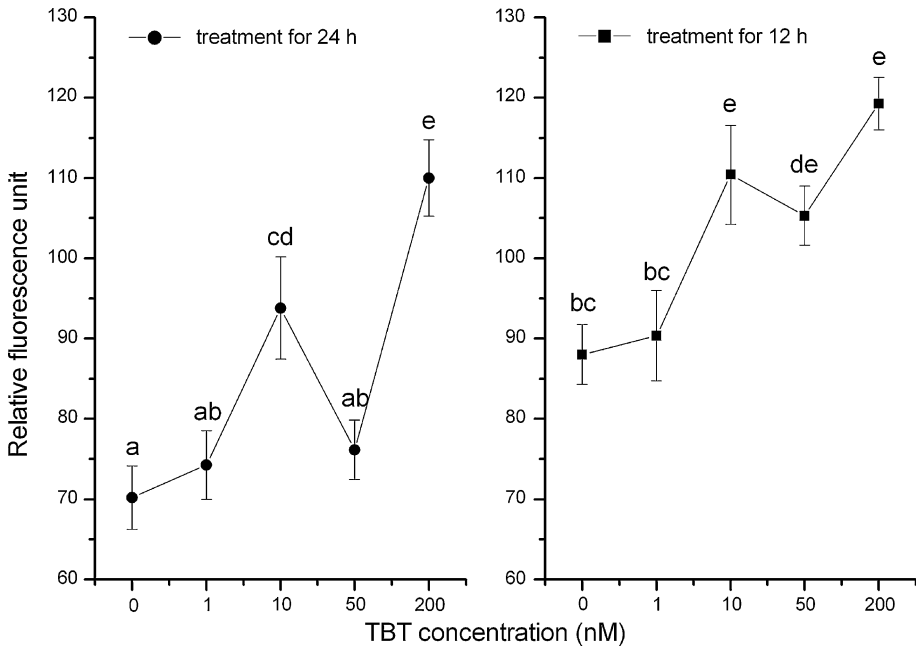

Fig. 3 TBT exposure caused dose-dependent increase in SOD-3 expression in head of the worm. All values are represented as mean ± SE, n = 15. Treatments not sharing a common letter are significantly different at $p < 0.05$

to nucleus translocation with the increase of TBT concentration. In a total of 150 worms, only 18.5 % worms underwent DAF-16 translocation under normal culturing conditions. Under 200 nM of TBT exposure, the percentage of DAF-16 translocation increased to 46.9 %, nearly 2.54 times higher compared to that of the control.

The nematode *C. elegans* is a good model for stress response and aging, drug screens, genotoxic stress and environmental toxicological studies (Ankeny 2001; Leung et al. 2008). The *C. elegans* is a free-living worm that survives in terrestrial, benthic, and aquatic environments, where it can be exposed to many kinds of environmental toxicants such as heavy metals or agricultural pesticides. It has a short lifespan, high fertility and it is easy to culture in laboratory, thereby producing many offspring in a short period of time and thus reducing test costs. Moreover, the fully sequenced genome in which 60 % of the genes have vertebrate counterparts, forward and reverse genetic tractability provides powerful tools to investigate the mechanisms of toxicants at organism level. Also, there are plentiful transgenic strains that carry GFP reporter genes, which provide us with powerful tools to analyze the expression of many proteins under various environmental settings.

It has been shown that TBT exposure caused oxidative stress in several experimental systems. In living organisms, ROS is generated under physiological conditions and is important for normal cell signaling. However, excessive

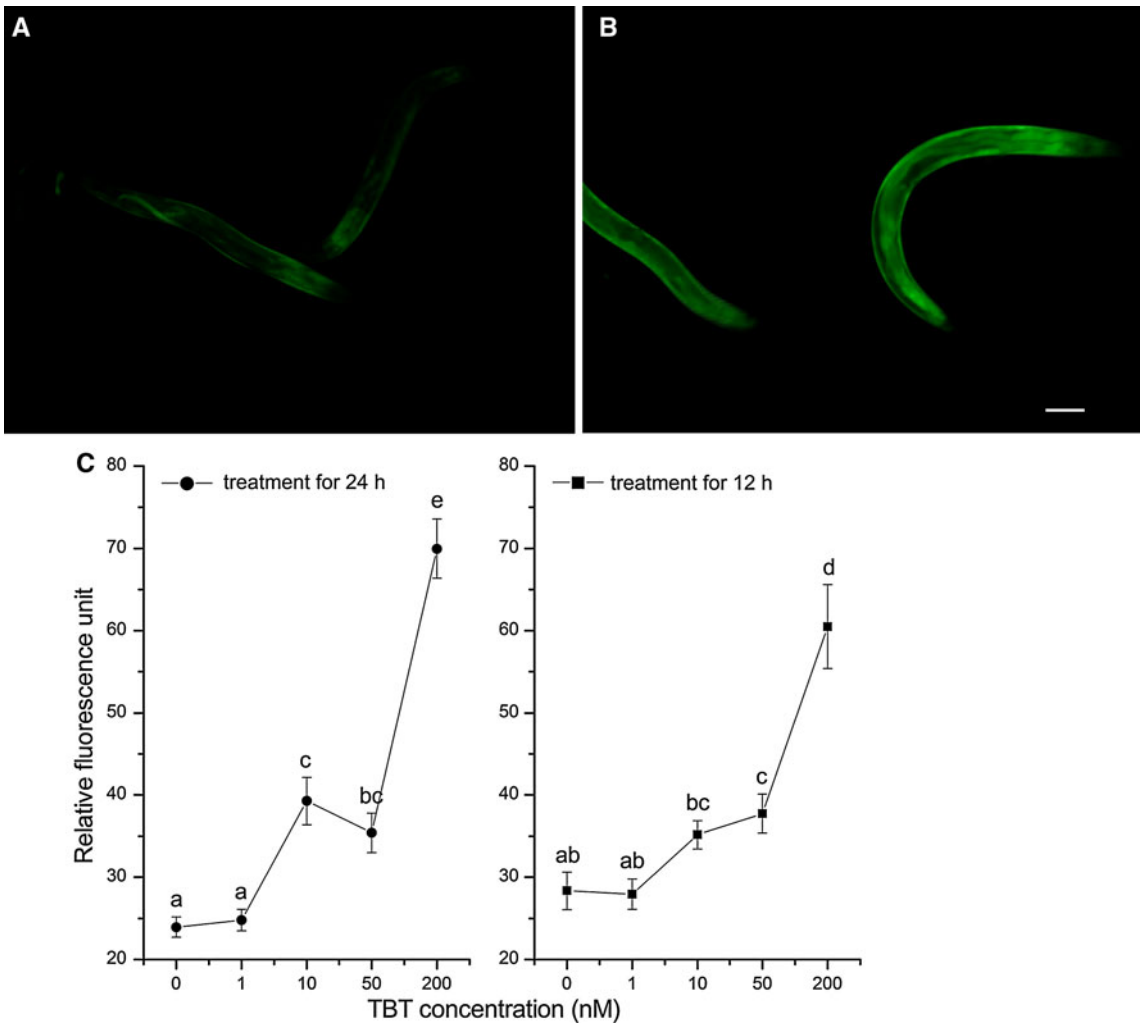

Fig. 4 The expression of GST-4 in the pharynx region and intestine of the worm was induced by TBT exposure. **a**, **b** Representative microphotographs of GST-4 expressions. *Bar* represents 0.1 mm. **a** Control or **b** with 200 nM of TBT. **c** TBT exposure caused dose-dependent increase in GST-4 expression. All values are represented as mean ± SE, n = 15. Treatments not sharing a common letter are significantly different at $p < 0.05$

ROS release could affect multiple cell processes and result in oxidative damage or cell death. By exposing *C. elegans* to different doses of TBT for 24 h, our data demonstrated that TBT exposure induced the release of ROS even at very low doses, resulting in DNA damage and the expression of stress response proteins. We examined the worms under physiological and TBT-exposing conditions for the localization of HUS-1, a component of the 9-1-1 DNA damage sensor complex, using worms expressing HUS-1::GFP (Hofmann et al. 2002). Our analyses have revealed that HUS-1::GFP foci numbers increased significantly at the concentrations of 50 and 200 nM TBT for 24 h exposure comparing to that of the control ($p < 0.05$). Thus, TBT-induced oxidative damage and DNA damage were confirmed in the worms in vivo.

SOD and GST play important roles in eliminating the excessive ROS generated by normal metabolism or under oxidative stresses (Fridovich 1997; Storey 1996). In *C. elegans*, *sod-3* encodes an iron/manganese superoxide dismutase that might defend against oxidative stress and promote normal lifespan. The *C. elegans gst-4* encodes a putative glutathione-requiring prostaglandin D synthase and is required for sperm to normally migrate towards a PUFA-based signal exuded by oocytes. It was reported that SOD activity significantly decreased with 0.05 and 0.1 μm TBT for 1 h exposure on haemocytes in the clam *Tapes Philippinarun* in vitro (Matozzo et al. 2002). Moreover, after Sprague–Dawley rats were exposed to 0.1–10 mg/kg of TBT, hepatic SOD activity increased with dosage after 3 days of exposure and decreased with dosage after 7 days of exposure (Liu et al. 2006). Previous studies indicated that exposing to TBT cause induction effects at lower doses and depletion effects at high doses for SOD and GST activities in marine fish (Wang et al. 2005). In the present

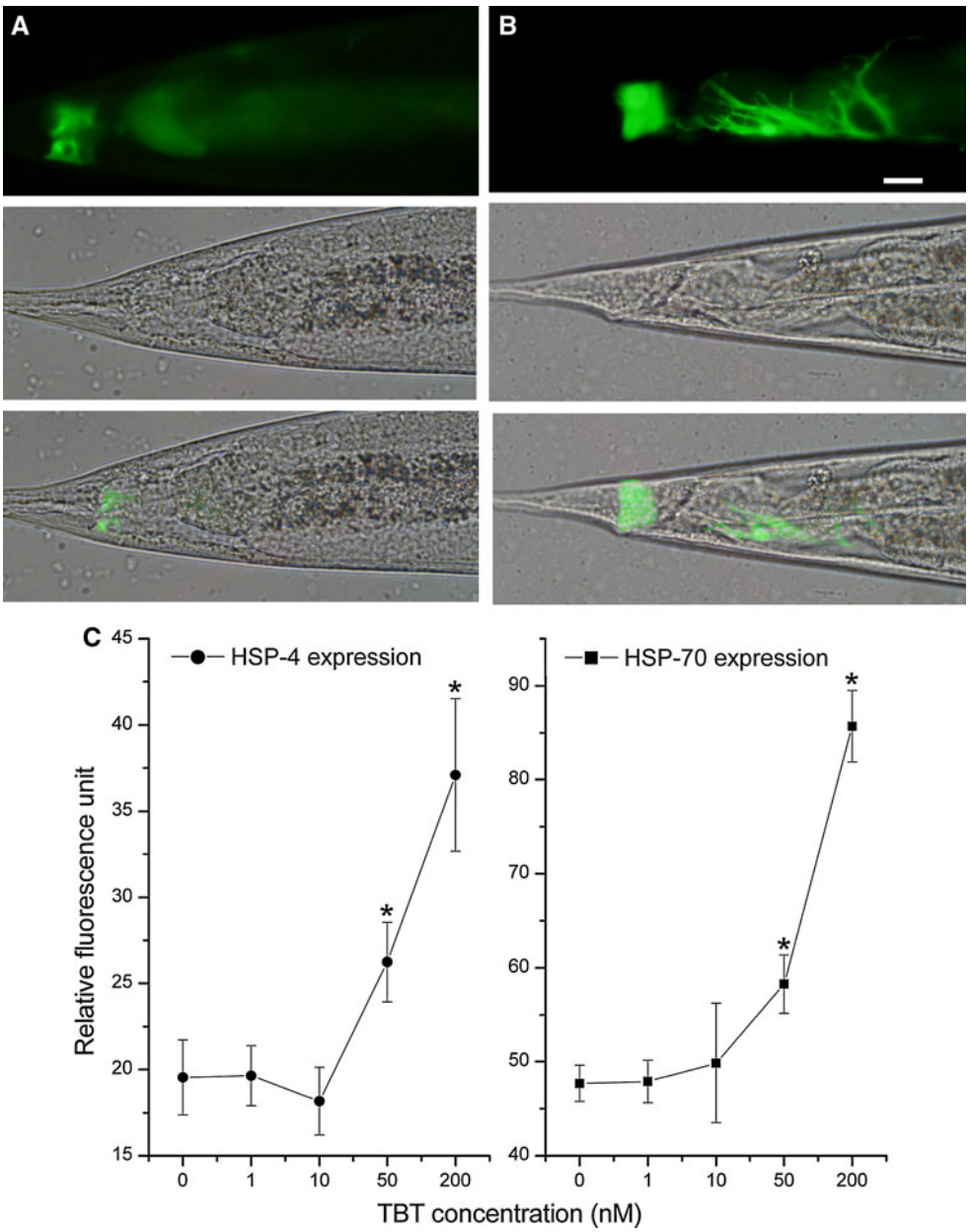

Fig. 5 The expressions of HSP-4 and HSP-70 were induced by TBT exposure. **a**, **b** Representative microphotographs of HSP-70 expressions at the posterior of the body in *C. elegans. Bar* represents 10 μm. **a** Control or **b** with 200 nM of TBT. **c** Quantification of HSP-4 and HSP-70 induced by TBT exposure. All values are represented as mean ± SE, n = 15. *Error bar* with *asterisk* represents statistical significance ($p < 0.05$) compared to that of the control

study, under TBT stress, the *C. elegans* SOD-3 and GST-4 were induced above the dosage of 10 nM. However, SOD-3 expression exhibited significant decline tendency ($p < 0.05$) by 10–50 nM TBT for 24 h exposure. Therefore, it is possible that activities of these detoxifying enzymes exhibit hormesis and debilitation effects at lower sublethal doses for animals exposed to environmental stresses such as organotin compounds.

The evolutionary conserved HSPs usually act as molecular chaperones, and play diverse roles in transporting, folding, assembling of degraded or misfolded proteins. Many researches indicated that HSPs played important roles in stress response and have been widely used as biochemical markers of environmental stress, such as toxicant exposure, both in vitro and in vivo (Iwama et al. 1998). In *C. elegans*, *hsp-4* transcription is up-regulated in response to endoplasmic reticulum stress induced by dithiothreitol (DTT) or tunicamycin as well as in response to heat shock. The *C. elegans hsp-70* encodes a heat-shock protein that is a member of the HSP-70 family of molecular chaperones. It has been reported that TBT can induce several HSPs expression such as HSP-60, HSP-63, HSP-70 and HSP-89 in mussel, crab and human (Oberdörster et al. 1998; Clayton et al. 2000; Zhang and Liu 1992). Our results indicated that TBT exposure not only induced the expression of HSP-70 family, but also increased the expression of small molecular HSP-4. However, in this work we also demonstrated that expression of the

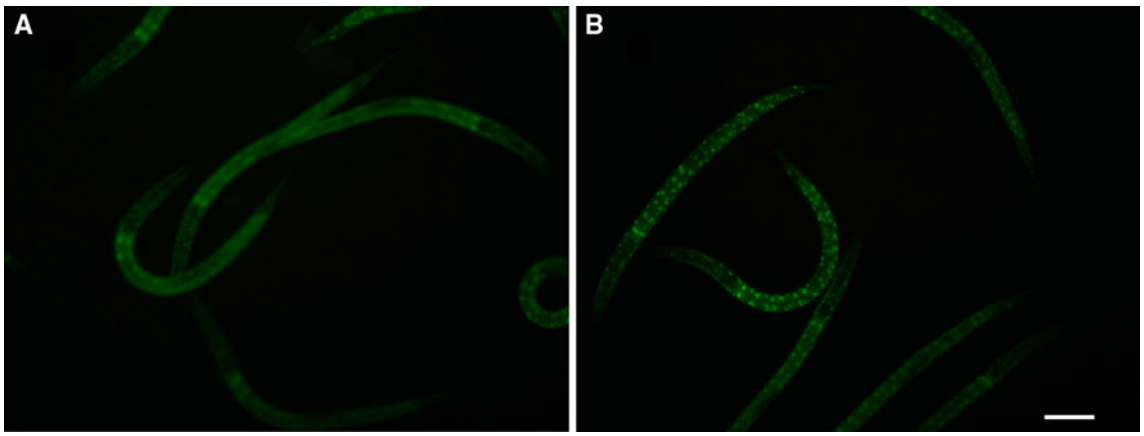

Fig. 6 Representative microphotographs of DAF-16 translocation from cytoplasm to nucleus in L2-L3 larva. **a** Control, **b** 200 nM of TBT. *Bar* represents 50 μm

Table 1 TBT induced DAF-16 translocation from cytoplasm to nucleus in the *C. elegans*

TBT concentration (nM)	Total worms observed	Percent of translocation (%)
0	150 ± 7	18.5 ± 3.2
1	165 ± 8	32.2 ± 5.8*
10	211 ± 9	34.2 ± 4.5*
50	146 ± 8	37.0 ± 6.1*
200	162 ± 5	46.9 ± 8.3*

* Represents statistical significance ($p < 0.05$) compared to that of the control

hsp-16.2::GFP reporter gene was not induced by TBT stress in the *C. elegans* strain CL2070 or TJ375. The results described here suggested that HSP-70 and HSP-4 played more important roles than HSP-16.2 in the *C. elegans* to protect against TBT stress.

The FOXO transcription factor DAF-16 is the major target of insulin-like signaling pathways, which promotes wild type lifespan, stress resistance and reproductive development in the *C. elegans* (Jensen et al. 2006). The DAF-16 protein normally lies in the cytoplasm, under adverse environmental stress, DAF-16 will translocate from cytoplasm to nucleus. In the nucleus, DAF-16 is known to activate transcription of a large number of genes that increase stress resistance and longevity. The inhibition of *daf-2*, a homologue of the insulin receptor (Zamore et al. 2000), also leads to nuclear localization of DAF-16::GFP (Henderson and Johnson 2001). Our present results indicated that TBT stress increased the rate of DAF-16 translocation to nucleus even at a very low concentration in the *C. elegans.* This can be explained that the nuclear localization of DAF-16::GFP may play a important role in activation of a series of genes encoding antioxidative proteins like SOD, GST and HSPs.

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 20907016 and No. 21077040). We would like to thank Caenorhabditis Genetics Center for providing *C. elegans* strains.

Conflict of interest We declare that there is no conflict of interests.

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