Effects of Tributyltin and Diazinon on the Intertidal Marine Harpacticoid Copepod Tigriopus japonicus

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Effects of Tributyltin and Diazinon on the Intertidal Marine Harpacticoid Copepod *Tigriopus japonicus*

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The intertidal zone plays an important role in the ecology of coastal waters by providing marine organisms a place to feed, grow, and spawn. Because the organisms that inhabit these areas may be contaminated and adversely affected by pollutants, an assessment of the impact of pollutants on intertidal zone organisms is needed. Tributyltin and diazinon are environmental pollutants that have been detected in coastal waters and are well known to be highly toxic to aquatic organisms. In this study, we performed acute and life–cycle toxicity tests with an intertidal organism, the copepod *Tigriopus japonicus*. The 48 h–LC50 (i.e., the concentration that killed half the organisms in 48 h) values for tributyltin revealed that nauplii (48 h–LC50; 3.72 μg/L) were more sensitive to tributyltin than adult males and gravid females (48 h–LC50; 7.16 and 7.69 μg/L, respectively). In contrast, the 48 h–LC50 values for diazinon showed that adult male and gravid females (48 h–LC50; 164 and 216 μg/L, respectively) were more sensitive to diazinon than nauplii (48 h–LC50; >1400 μg/L). Life–cycle toxicity tests with tributyltin or diazinon showed that tributyltin and diazinon reduced the fecundity of *T. japonicus* under the experimental conditions. The lowest concentrations of tributyltin and diazinon that reduced the fecundity of *T. japonicus* were found to be 1.6 μg/L and 15.7 μg/L, respectively. Although the risk of exposure to tributyltin and diazinon individually in the environment was estimated to be negligible, a mixture of the two might exert adverse effects on *T. japonicus* in the intertidal zone.

Key words: diazinon, intertidal zone, life–cycle toxicity test, *Tigriopus japonicus*, tributyltin

INTRODUCTION

A variety of pollutants that are toxic to marine organisms have become a concern because of their impact on marine ecosystems (Beiras, 2018). Xenobiotics such as antifouling paints and pesticides have contaminated some aquatic ecosystems. Pollutants that are discharged directly into coastal waters or delivered via streams eventually reach the intertidal zone. A wide range of chemical pollutants, including polycyclic aromatic hydrocarbons (PAHs) (Tipmanee *et al*., 2012), persistent organic pollutants (POPs) (Fu *et al*., 2003), and heavy metals (Zhou *et al*., 2007), can be introduced into coastal waters. In previous studies, a variety of pollutants (e.g., tributyltin, radioisotopes, and PAHs) have been detected in wharf roaches (*Ligia* sp.) collected from the coastal zone (Honda *et al*., 2018; Qiu *et al*., 2017; Undap *et al*., 2013a). In addition, heavy oil spilled from tankers such as the *Torrey Canyon*, *Nakhodka*, and *Prestige* has caused severe impacts on coastal environments (Chen *et al*., 2019). In coastal waters, the intertidal zone plays an important role by providing places for marine organisms to feed, grow, and spawn (Gibson, 2001). Organisms that inhabit coastal waters are therefore likely to be contaminated by and adversely affected by pollutants.

Tributyltin (TBT) is a highly toxic chemical that has been used worldwide in antifouling paints, mainly on ships and aquaculture facilities, to prevent the growth of marine organisms such as bacteria, mussels, and algae (Batley and Scammell, 1991; Cardwell *et al*., 1999; Ko *et al*., 1995; Murai *et al*., 2005). It is well known that TBT severely disrupts the endocrine systems of marine organisms, and impaired sexual development is among the typical and severe impacts of TBT on marine organisms (Horiguchi *et al*., 1997; Matthiessen and Gibbs, 1998; Shimasaki *et al*., 2003). Although use of TBT is strictly regulated because of its potent toxicity to non–target organisms, a number of countries have not yet ratified the control of TBT for shipping activities (IMO, 2020), and TBT is still detected in coastal areas (Sheikh *et al*., 2020; Undap *et al*., 2013b). Because TBT is adsorbed on floating objects such as plastic debris (Carter *et al*., 1989) and on aquatic microorganisms (Gadd, 2000), it is likely to reach and be widely distributed in the intertidal zone, where it may affect intertidal

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organisms.

Diazinon (O,O–diethyl–O–[2–isopropyl–6–methyl–4–pyrimidinyl] phosphoro thioate) is an organophosphate insecticide that is used in agricultural fields worldwide (Bailey *et al*., 2000). Its irreversible inhibition of cholinesterase is known to be primarily responsible for the toxicity of diazinon, and it is also well established that diazinon is highly toxic to vertebrate animals as well as insects (Davies and Holub, 1980; Keizer *et al*., 1995; Nakagawa and Moore, 1999). Diazinon has been detected mainly in agricultural fields (Góngora–Echeverría *et al*., 2019; Ngolo *et al*., 2019), but it has also been detected in rivers and coastal areas (Derbalah *et al*., 2019; Köck–Schulmeyer *et al*., 2019). It has therefore been suggested that diazinon may be affecting organisms in the intertidal zone.

Harpacticoid copepods such as *Tigriopus sp*. have been widely used as model organisms in eco–toxicity tests (Barka *et al*., 2001; O'Brien *et al*., 1988). *Tigriopus japonicus* is a marine harpacticoid copepod that lives in the intertidal zone around East Asia; it has been widely used as a marine model organism in many scientific studies (Marcial *et al*., 2003; Rhee *et al*., 2009). *Tigriopus japonicus* is easily maintained under artificial conditions. The well–known ontogeny and life cycle of *T. japonicus* include six naupliar stages and five copepodid stages before *T. japonicus* reaches adulthood and sexual maturity. Furthermore, *T. japonicus* males can be easily distinguished from females based on their first antenna and fifth swimming legs. These characteristics make *T. japonicus* a model organism well suited for observation of the effects caused by chronic exposure to chemical substances (Raisuddin *et al*., 2007). In the intertidal zone, risk analysis studies with *T. japonicus* have been performed mainly with pollutants such as TBT (Kwok and Leung, 2005) and oil (Lee *et al*., 2013). However, few risk analysis studies of pesticide toxicity to *T. japonicus* have been performed. To evaluate the environmental risks of TBT and diazinon in the intertidal zone, we performed acute toxicity tests and life–cycle toxicity tests with *T. japonicus* exposed to TBT and diazinon.

MATERIALS AND METHODS

Harpacticoid copepod

The harpacticoid copepod *T. japonicus* was obtained from the Marine Ecology Research Institute (Chiba, Japan), and maintained in culture in our laboratory. *T. japonicus* was cultured in 2–L glass beakers with artificial seawater (salinity of 30, dissolved oxygen > 6 mg/L, 25 ± 1°C) (Marine Art SF–1, Tomita Pharmaceutical, Tokushima, Japan). *T. japonicus* was maintained on a 12 h:12 h (light:dark) cycle of illumination and fed the green alga *Tetraselmis tetrathele*, which was cultured in modified SWM–3 medium (Itoh and Imai, 1987).

Chemical substances

Tributyltin (Tri–*n*–butyltin oxide, > 95% pure) was purchased from the Tokyo Chemical Industry (Tokyo, Japan). The TBT was dissolved in ethanol to prepare a stock solution of 300 μg/mL. The stock solution was stored in the dark at 4°C. The ethanol concentrations in the test solution was < 0.1 mL/L.

Diazinon (diethyl 2–isopropyl–4–methyl–6–pyrimidinyl phosphonothioate, 99% pure), was purchased from Wako Pure Chemical (Tokyo, Japan). The diazinon was dissolved in dimethylformamide (DMF) to prepare a stock solution of 14 mg/mL. The stock solution was stored in the dark at 4°C. Test solutions were prepared by pipetting calculated amounts of the stock solutions into known volumes of the artificial seawater. The DMF concentrations in the test solution were < 0.1 mL/L.

Determination of TBT and diazinon concentrations in test solutions

TBT and diazinon concentrations in the test solutions were verified analytically with a gas chromatograph equipped with a mass spectrometer (GC/MS) at the beginning of the exposure tests. Analyses of TBT concentrations in the test solutions were performed as previously described (Inoue *et al*., 2006). The TBT concentrations were analyzed using a GC/MS (model 6890 gas chromatograph, model 5973 mass spectrometer, Hewlett–Packard, California, USA). The dissolved diazinon was extracted from 1–L samples in a solid–phase extraction cartridge (Sep–Pak, Waters Corporation, Massachusetts, USA) and then eluted with 5 mL of dichloromethane. The eluate was concentrated to 1 mL and analyzed using GC/MS. The concentrations of TBT and diazinon reported here are the concentrations that we measured in this way.

48–h acute toxicity tests

Acute toxicity tests were performed to estimate the concentrations that killed 50% of the test organisms in 48 h (48 h–LC50) using nauplii (< 24 hours old), adult male, and gravid female (9 days old) copepods. Exposure concentrations of TBT were 1.78, 5.65, 7.82, 12.4, 24.4 μg/L, and a solvent control (ethanol, 0.01% [v/v]) for nauplii and adult copepods. Exposure concentrations of diazinon were 1.4, 14, 140, 1400 μg/L, and a solvent control (DMF, 0.01% [v/v]) for nauplii; and 19.5, 35.6, 73.1, 142, 326 μg/L, and a solvent control (DMF, 0.01% [v/v]) for the adult copepods. These exposure concentrations were selected based on preliminary exposure tests. Eight nauplii were transferred by Pasteur pipette to 5–mL glass Petri dishes containing 5 mL of test solution at each concentration, and 8 adult male or gravid female copepods were transferred by glass pipette to 100–mL glass beakers containing 100 mL of test solution at each concentration. After exposures of 24 h and 48 h, the mortalities of the copepods were assessed. Copepods were not fed during the exposure period. The experimental cultivation conditions were otherwise the same as the normal cultivation conditions.

Life–cycle toxicity tests

Life–cycle toxicity tests were performed as

described in previous studies (Hutchinson *et al*., 1999; Marcial *et al*., 2003) but with a slight modification. In the life–cycle toxicity test for TBT, experimental treatments consisted of a control, solvent control (ethanol, 0.01% [v/v]), and four concentrations of TBT (0.45, 0.88, 1.6, and 3.9 μg/L). In the life–cycle toxicity test for diazinon, the treatments consisted of a control, solvent control (DMF, 0.01% [v/v]), and five concentrations of diazinon (7.73, 15.7, 33.1, 73.6, and 147 μg/L). These concentrations were selected based on 48–h acute toxicity tests.

Five nauplii (< 24 hours after hatching) were transferred into a 5–mL glass Petri dish filled with 4 mL of test solution. There were four replicates in each group. The exposure period was set to 16 days. The test solution contained *T. tetrathele* (1×10^5 cells/mL) and was renewed every two days during the exposure period. Survival and the developmental stage of the copepods were observed every 24 h using a stereo microscope (SMZ, Nikon, Tokyo, Japan). After a 6–d exposure, all surviving copepods from each test concentration were transferred to 100–mL glass beakers containing 20 mL of test solution with *T. tetrathele* to initiate copulation. After 2 or 3 days, all gravid females, each bearing an ovisac, were individually transferred into 5–mL glass Petri dishes. After the gravid females had spawned, the number of nauplii was counted for each copepod. The cultivation conditions during this test were the same as normal cultivation conditions (salinity of 30, dissolved oxygen > 6 mg/L, 25 ± 1˚C, 12 h:12 h light:dark cycle of illumination).

Statistical analysis

48 h–LC50 values for each chemical were determined with a logistic regression model (Piegorsch and Bailer, 2005). Calculations were performed with R software (version 3.6.2, https://cran.r–project.org/). The 48 h–LC50 estimates were based on the measured concentrations of each chemical. The differences of the survival rates between the control and exposure groups were analyzed with a log–rank test in R. The differences in fecundity and the number of days required to attain the copepodid stage and to become gravid were analyzed by Dunnett's test in R. Differences in sex ratios were analyzed by two–sample tests for equality of proportions in R. A type I error rate (p–value) less than 0.05 was regarded as being significant for all tests.

RESULTS

48 h–LC50

The 48 h–LC50 values for TBT were higher in adult males and gravid females (7.16 and 7.69 μg/L) than in nauplii (3.72 μg/L) (Table 1). The 48 h–LC50 values for diazinon were higher in nauplii (> 1400 μg/L) than in adult males and gravid females (164 and 216 μg/L, respectively) (Table 1).

Survival rate in life–cycle toxicity tests

In the TBT treatment group, the survival rate of *T. japonicus* was unaffected by TBT concentrations ≤1.6 μg/L. In the group exposed to 3.9 μg/L TBT, the survival rate at 6 days decreased from 90–100% to 65% (Fig. 1A). The survival rates of copepods in the diazinon treatment group were unaffected by concentration ≤73.6 μg/L. At 14 days, however, the mortality of copepods exposed to 147 μg/L diazinon was 100% ($p < 0.01$, Fig. 1B).

Table 1. Acute toxicities (48 h–LC50 s) of TBT and diazinon to *T. japonicus*

	48 h–LC50 (μg/L)		
	Nauplius	Adult male	Gravid female
TBT	3.72	7.16	7.69
Diazinon	>1400	164	216

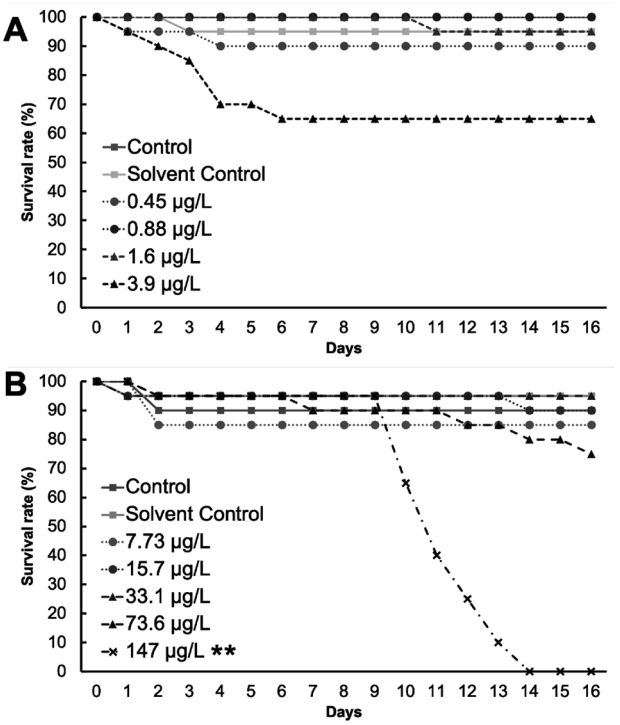

Fig. 1. The survival rate of *Tigriopus japonicus* in the life–cycle exposure test.
A. The survival rate in the TBT exposure group. **B**. The survival rate in the diazinon exposure group. Significant differences versus the control group are indicated by asterisks (**) that indicate $p < 0.01$ (log–rank test).

The effects of TBT on the life–cycle of *T. japonicus*

The elapsed time from hatching to the copepodid stage was delayed from 4 days to 4.4 days in the group exposed to 3.9 μg/L TBT ($p < 0.05$, Table 2). The elapsed time from hatching to maturation was significantly delayed from 10 days to 11 days or 15 days in the groups exposed to 1.6 μg/L or 3.9 μg/L TBT, respectively ($p < 0.05$, Table 2). No significant differences were found in the sex ratios of the control group and exposure

Table 2. The elapsed time for development and fecundity of *T. japonicas*

TBT	Nauplius stage[a] (days)	Maturation stage[b] (days)	Sex ratio[c] (female/male)	Fecundity[d] (nauplii/female)
Control	4.1 ± 0.2	10.0 ± 0.6	6/14 (0.4)	76.8 ± 21.3
Solvent control	3.7 ± 0.5	9.3 ± 0.5	4/15 (0.3)	84.5 ± 14.9
0.45 μg/L	4.0 ± 0.0	10.3 ± 0.7	12/6 (2.0)	55.5 ± 29.9
0.88 μg/L	4.0 ± 0.0	10.7 ± 0.7	10/10 (1.0)	53.3 ± 36.9
1.6 μg/L	4.0 ± 0.0	11.0 ± 0.7*	9/10 (0.9)	45.9 ± 32.2
3.9 μg/L	4.4 ± 0.7*	15.0 ± 1.4*	2/11 (0.2)	2.0 ± 2.8*
Diazinon	**Nauplius stage[a] (days)**	**Maturation satage[b] (days)**	**Sex ratio[c] (female/male)**	**Fecundity[d] (nauplii/female)**
Control	3.8 ± 0.4	9.2 ± 0.4	6/12 (0.5)	72.8 ± 14.3
Solvent control	3.9 ± 0.5	9.1 ± 0.6	8/11 (0.7)	79.8 ± 14.1
7.73 μg/L	3.6 ± 0.6	9.6 ± 2.0	8/9 (0.9)	63.9 ± 36.8
15.7 μg/L	3.8 ± 0.6	9.8 ± 0.8	5/14 (0.4)	34.8 ± 23.1*
33.1 μg/L	4.1 ± 0.2	9.3 ± 0.9	9/10 (0.9)	1.0 ± 3.0*
73.6 μg/L	3.8 ± 0.8	9.5 ± 0.7	6/9 (0.7)	0*
147 μg/L	4.0 ± 0.0	–	–	0*

Significant changes versus control group ($p < 0.05$) are indicated by an asterisk (*). (a) The time from hatching to reaching the copepodid stage. (b) The time from female birth to bearing an ovisac. (c) The sex ratio of adult copepods. (d) The number of nauplii produced by one female copepod.

groups (Table 2). Copepod fecundity was dramatically decreased in the group exposed to 3.9 μg/L TBT ($p < 0.05$, Table 2). In the control group, 76 nauplii were produced per female, but only 2 nauplii were produced per copepod in the group exposed to 3.9 μg/L TBT. Based on the results of copepod TBT–exposure tests, the no observed effect concentration (NOEC) was estimated to be 0.88 μg/L, and the lowest observed effect concentration (LOEC) was estimated to be 1.6 μg/L. The predicted no–effect concentration (PNEC) of TBT was calculated from the NOEC with an assessment factor (AF) of 100 and was estimated to be 8.8 ng/L (3.6 ng Sn/L) (ECC, 2003).

The effects of diazinon on the life–cycle of *T. japonicus*

In the diazinon treatment group, all copepods in the group exposed to 147 μg/L diazinon were dead before they bore ovisacs (Fig. 1B). There was no significant effect of diazinon exposure on the elapsed time for copepod development or on the sex ratio (Table 2). However, there was a significant decrease of fecundity in the groups exposed to 15.7, 33.1, 73.6, and 147 μg/L diazinon ($p < 0.05$, Table 2). In the control group, approximately 72 nauplii were produced per female . However, only 0–1 nauplii per female were produced in the groups exposed to diazinon at concentrations of 33.1–147 μg/L (Table 2). In the group exposed to 73.6 μg/L diazinon, no gravid females produced nauplii, even though females bore ovisacs. Based on the results of diazinon exposure to copepods, the NOEC and LOEC were estimated to be 7.73 μg/L and 15.7 μg/L, respectively. The PNEC of diazinon was calculated from the NOEC with an AF of 100 and was estimated to be 77.3 ng/L (ECC, 2003).

DISCUSSION

The 48–h acute toxicity tests revealed that nauplii were more sensitive to TBT than adult copepods, i.e., the 48 h–LC50 was lower for nauplii (3.72 μg/L) than for adult male and gravid female copepods (7.16 and 7.69 μg/L, respectively). This result was consistent with the results of previous studies on other harpacticoid copepods; those studies have demonstrated that nauplii are more sensitive to toxic chemicals than copepodids or adult copepods (Guo *et al*., 2012; Verriopoulos and Moraïtou–Apostolopoulou, 1982).

In the life–cycle toxicity tests with TBT, all copepods exposed to 3.9 μg/L TBT developed from hatching to copepodids in about 5 days. The survival rate decreased during 6 days of exposure to 3.9 μg/L TBT (Fig. 1A) but was then stable until the end of the exposure test. This result was in accord with the results of previous studies of the acute toxicity of TBT, which have demonstrated that the tolerance of *T. japonicus* to TBT increases through ontogenetic development from nauplius to copepodid (Guo *et al*., 2012; Verriopoulos and Moraïtou–Apostolopoulou, 1982).

In contrast, the results of the diazinon 48–h acute toxicity tests demonstrated that adult copepods were more sensitive to diazinon than nauplii, i.e., the 48h–LC50 was higher for nauplii (> 1400 μg/L) than for adult male and gravid female copepods (164 and 216 μg/L, respectively). This result agreed well with the results of the previous study of Rompas *et al*. (1989), which demonstrated that tiger shrimp post–larvae are more sensitive to an organophosphate pesticide (fenitrothion) than

nauplii. Kobayashi *et al*. (1990) have also indicated that the metabolic activity of tiger shrimp larvae exposed to the thiono–form and oxo–form of the organophosphate in fenitrothion increases as the larvae grow. They have demonstrated that the oxo–form is more toxic to the larvae than the thiono–form. In this study, the survival rate of *T. japonicus* exposed to 147 μg/L diazinon began to decrease significantly after maturation to the adult copepod stage. These results suggested that tolerance to diazinon decreased during ontogenetic development from nauplius to adult copepod. We therefore hypothesized that the metabolism of diazinon by *T. japonicus* increased during its growth and that an increase of the oxo–form of diazinon caused its high toxicity to adult *T. japonicus*.

The life–cycle toxicity tests revealed that copepods exposed to 73.6 μg/L diazinon or 3.9 μg/L TBT could not produce nauplii, even though gravid females bore ovisacs. These results indicated that diazinon and TBT inhibit and/or delay the production of nauplii. In contrast, Lee *et al*. (2008) have shown that exposure to 10 μg/L TBT does not affect the fecundity of *T. japonicus*. In this study, we used a glass Petri dish and beaker as a test chamber and estimated the 48 h–LC50 of TBT for adult copepods to be 7.16 μg/L. In contrast, Lee *et al*. (2008) used a plastic culture plate as a test chamber and estimated the 96 h–LC50 of TBT for adult copepods to be 50 μg/L. The differences of test chamber material may have contributed to a difference of TBT concentrations in the test chambers.

In recent studies, TBT has been detected at a maximum concentration of 2.2 ng/L (0.9 ng Sn/L) in seawater sampled from the port of Zanzibar City in Tanzania (Sheikh *et al*., 2020), and diazinon has been detected at a maximum concentration of 0.71 ng/L in seawater sampled from Spanish coastal waters (Köck–Schulmeyer *et al*., 2019). These concentrations are lower than the PNEC of TBT (8.8 ng/L (3.6 ng Sn/L)) and diazinon (77.3 ng/L), respectively, in this study. The corresponding hazard ratios are 0.25 (0.9/3.6) for TBT and 0.009 (0.71/77.3) for diazinon. These results suggest low or negligible risk of TBT and diazinon in the coastal zone. In the intertidal zone, however, TBT as well as radionuclides and PAHs have been detected in wharf roaches (Honda *et al*., 2018; Qiu *et al*., 2017; Undap *et al*., 2013a). Thus, intertidal organisms may be exposed to a mixture of pollutants. The additive behavior of the toxicity of pollutants has been well documented, and Qiu *et al*. (2019) have shown that a mixture of TBT and polychlorinated biphenyls (PCBs) can be toxic to medaka embryos even if there is no toxicity to medaka embryos associated with exposure to the same concentrations of TBT or PCBs individually. We therefore suggest that further research will be required to evaluate the toxicity of combinations of chemical pollutants to intertidal organisms such as *T. japonicus*.

In conclusion, we performed acute and life–cycle toxicity tests using *T. japonicus* and evaluated the effects of TBT and diazinon on the offspring of *T. japonicus* adults. Our results suggested that the risk of exposure to TBT or diazinon individually was negligible in the intertidal zone. However, several pollutants might be simultaneously present and contaminate intertidal organisms. The combined toxicity of several pollutants may adversely affect the intertidal zone ecosystem. Further research is therefore needed to evaluate the toxic effects of combinations of several pollutants on intertidal zone organisms such as *T. japonicus*.

AUTHOR CONTRIBUTIONS

Y. Takai designed the study, analyzed the data, and wrote the paper. W. Tanoue performed the acute and life–cycle toxicity tests and measured the TBT and diazinon concentrations in the test solutions. X. Qiu supervised the statistical analysis in the study. H. Takaku provided the *T. japonicus* that was used in this study. I. J. Kang supervised the work and assisted with writing the paper. Y. Shimasaki designed the study and supervised the work. T. Honjo and Y. Oshima designed the study, supervised the work, and provided facilities and resources. All authors assisted in editing the manuscript and approved the final version.

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