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Tributyltin toxicity in abalone (*Haliotis diversicolor supertexta*) assessed by antioxidant enzyme activity, metabolic response, and histopathology

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ABSTRACT

A toxicity test was performed to investigate the possible harmful effects of tributyltin (TBT) on abalone (*Haliotis diversicolor supertexta*). Animals were exposed to TBT in a range of environmentally relevant concentrations (2, 10 and 50 ng/L) for 30 days under laboratory conditions. TBT-free conditions were used as control treatments. The activity of antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD), and malondialdehyde (MDA), along with levels of haemolymph metabolites, and hepatopancreas histopathology were analyzed. The results showed that TBT decreased SOD activity, and increased POD level and MDA production in a dose-dependent way, indicating that oxidative injury was induced by TBT. Haemolymph metabolite measurements showed that TBT increased alanine and glutamate levels, and decreased glucose content, which suggested perturbation of energy metabolism. Elevated levels of acetate and pyruvate in the haemolymph indicated partial alteration of lipid metabolism. A decrease in lactate and an increase in succinate, an intermediate of the tricarboxylic acid (TCA) cycle, indicated disturbance of amino acid metabolism. Hepatopancreas tissues also exhibited inflammatory responses characterized by histopathological changes such as cell swelling, granular degeneration, and inflammation. Taken together, these results demonstrated that TBT was a potential toxin with a variety of deleterious effects on abalone.

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1. Introduction

With the increasing release of environmental contaminants into the water ecosystem, concerns have been raised about potential toxicity and danger to aquatic organisms. Pollutants such as endocrine disrupting compounds (EDCs) can adversely affect fish, shellfish, and other aquatic species [1,2], often disturbing reproductive, developmental, and endocrine functions [3]. Effectively evaluating the effect of EDC toxicity on ecologically relevant species requires new ways of clarifying the toxicological mechanisms of endocrine disruptors.

Antioxidant status is important for understanding EDC effects. A review by Bernanke and Köhler [4] states that EDCs cause increases in oxygen free radical levels, and disturb the antioxidative balance in wildlife vertebrates. Antioxidant enzymes such as superoxide dismutase (SOD) are important in mediating responses to EDCs stressors. Barreira et al. [5] demonstrated that oxidative damage was the main cause of reproduction failure in clams, after exposure to polycyclic aromatic hydrocarbons. Some researchers have also found that EDC-induced physiological dysfunction in aquatic organisms was related to several antioxidant enzymes, such as catalase, glutathione-s-transferase, and peroxidase (POD) [6,7]. A further study suggests that an increase in POD and generation of reactive oxygen species (ROS) may be involved in imposex under environmental contamination conditions [8].

Metabolites are low molecular weight intermediates whose production is context dependent and whose levels change with the physiological, developmental, or pathological state of cells, tissues, organs, or organisms [9]. Metabolic profile analysis has theoretical advantages over genetic or protein profiling, since the metabolic network is downstream of gene expression and protein synthesis; thus, metabolic investigations reveal cellular activity at a functional level [10]. Furthermore, unlike gene or protein techniques, metabolite assays do not strictly require that the genome sequence of the organism of interest be known, a feature that is particularly important for ecological species (*e.g.* gastropods), whose genome sequences are incomplete. Another advantage of metabolite profile analysis is that it is relatively less expensive than other "omic" techniques. Because of these advantages, metabolite profiling has been increasingly employed, with many studies reporting the physiological effect of pollutants or environmental stressors on ecologically important organisms by metabolic assay [11–16].

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In this study, we investigated the toxicological effects and mechanisms of tributyltin (TBT) on abalone (a typical gastropod) using enzyme activity and metabolite assays. TBT was selected because it is a worldwide pollutant that exists widely in marine ecosystems. It can persist in the environment for a long time, and potentially interferes with endocrine and immune response, and the reproductive functions of wildlife [17,18]. Despite its legislatively limited release in most countries, TBT contamination is still a problem in many areas, and its persistence results in a cumulative contamination of the aquatic environment [19]. Abalone was selected as the target organism in this study because of its wide existence in marine environments, its high sensitivity to environmental changes, and because it is easy to manipulate [20]. Although imposex reproductive toxicology has been investigated in abalone after TBT exposure [21], the biochemical mechanisms have not been sufficiently addressed. Here, we analysed haemolymph antioxidant enzyme activity and endogenous metabolites to investigate the possible harmful effects induced by chronic TBT stimuli.

2. Materials and methods

2.1. Animals, experimental design and sample preparation

Abalones (initial weight 10.4 ± 2.1 g; shell length 45.1 ± 3.7 mm) were supplied by a local abalone hatchery in Shenzhen, China. TBT (purity ≥ 98.0%) was purchased from Wako (Osaka, Japan). A TBT solution (dissolved in acetone) was added to seawater to final concentrations of 2, 10, and 50 ng/L, which is within the natural concentration of TBT in environmental conditions [22]. The final acetone concentration was the same for each treatment (0.005%). Acetone without TBT was used for control groups, and seawater without TBT was used as blanks. Each treatment was performed in duplicate. Experiments were carried out in glass aquaria (80 cm × 50 cm × 40 cm) with 30 healthy abalones in each container. The test solution and seawater were changed daily. Throughout the experiments, the temperature was 24–26 °C, salinity was 30–33%, pH was 7.8–8.2, and the concentration of dissolved oxygen was no less than 6 mg/L. Abalones were fed with the marine alga *Gracilaria tenuistipitata* for both the acclimation and experimental periods.

Five abalones from each group were randomly sampled after a 30-day exposure to treatment. Abalones were deprived of food 24 h before sampling to eliminate possible stress influences [23]. Up to 1 mL of haemolymph was collected from the foot muscle via sinus puncture, and centrifuged (5000 rpm, 20 min, 4 °C). The cell-free supernatant was collected, and precipitates removed by ultrafiltration membrane (0.45 μm). Haemolymph was preserved at −80 °C before antioxidant enzyme activity and metabolite analysis. After the 30-day exposure period, hepatopancreatic tissues were collected for histological assays.

2.2. Antioxidant enzyme activity assays

POD activity was measured following the manufacturer's instructions of a peroxidase assay kit (catalog: A084-1) (Jiancheng Bioengineering Institute, Nanjing, China) with 20 μL samples and 180 μL of color-developing buffer (7.3 g $C_6H_8O_7 \cdot H_2O$, 11.86 g $Na_2HPO_4 \cdot 2H_2O$, 1 L H_2O), in 96-well microtiter plates. Optical density $(OD)_{490}$ (A1) was read, and the 96-well microtiter plate was removed, and 20 μL color-developing reagents (4 mg $C_6H_8N_2$, 4 μL 30% H_2O_2, 10 mL color-developing buffer) added. Plates were shaken five times in a microplate spectrophotometer, and color developed for 15 min in the dark, before the OD_{490} (A2) was read. Relative POD activity was expressed as A2 − A1. Specific activity was as μmol/mg protein.

Malondialdehyde (MDA) content represented the lipid peroxidation level in abalone haemolymph. A thiobarbituric acid reaction method was used to determine MDA content, with MDA reacting with thiobarbituric acid to form a stable pink chromophoric production measured at 532 nm [24]. MDA content was expressed in nmol/mg protein.

SOD activity was measured using a commercial SOD assay kit (catalog: A001-1) (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. A 10 μL aliquot of haemolymph was placed in a microplate containing 200 μL of reaction mixture, and 50 μL of xanthine oxidase solution was added. OD was measured at 505 nm and 37 °C using a microtiter plate reader (Thermo, arioskanFlash, USA). Reaction rate was estimated from absorbance readings at 1 and 5 min after addition of xanthine oxidase. A reference standard of SOD was supplied with the kit. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine oxidase reaction. SOD activity was expressed as U/mg protein.

2.3. Metabolism analysis

Haemolymph metabolites glucose, glutamate, alanine and succinate were determined using commercially available kits (Fenghui Medical Science Tech. Co., Ltd., Shanghai, China), specifically a glucose monitoring kit (catalog: TD-4223A), a glutamate assay (catalog: K692-100), an alanine assay (catalog: K652-100), and a succinate assay kit (catalog: JKY/245811). Biochemical parameters were analyzed with an automatic analyzer (Olympus AU 2700, Japan) using the analytic program.

The analysis of organic acids used pyruvate, lactate, and acetate standards from Supelco (Bellefonte, PA, USA). Ultrapure water was prepared using a Thermo Scientific EASYpure RODi System (USA). All other reagents were of analytical grade. Haemolymph samples were filtered through a 0.45-μm cellulose membrane before chromatographic analysis on a high-performance liquid chromatography system (Tosoh G7 Automated HPLC Analyzer) with a TSK-gel G or HSi column (7.5 mm × 300 mm) with a particle size of 5 μm (Tosoh Bioscience, Minato-Ku, Japan). Analyses were carried out isocratically at a flow rate of 0.5 mL/min, with mobile phase water adjusted to pH 2.1 with metaphosphoric acid. The column was thermostated at 30 °C, and injection volume was 20 μL. Organic acids were detected at 210 nm. Pyruvate, lactate and acetate were identified by retention and spectral data. The total time between injections was 40 min.

The protein concentration of haemolymph was measured using the Bradford method (1976) with bovine serum albumin (Sigma) as a standard. Each sample was tested in duplicate.

2.4. Histological examination

Hepatopancreas samples were fixed in 10% neutral formalin for more than 24 h, and were dehydrated in a graded ethanol series, and embedded in paraffin blocks according to standard methods [25]. Sections were cut at 7 μm and stained with hematoxylin and eosin (HE). Slides were examined by light microscope for routine histology and morphometrics, and histological measurements were taken using an ocular micrometer.

2.5. Statistical analysis

Data are expressed as mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) with SPSS 11.0 software. Differences were considered significant at $P < 0.05$.

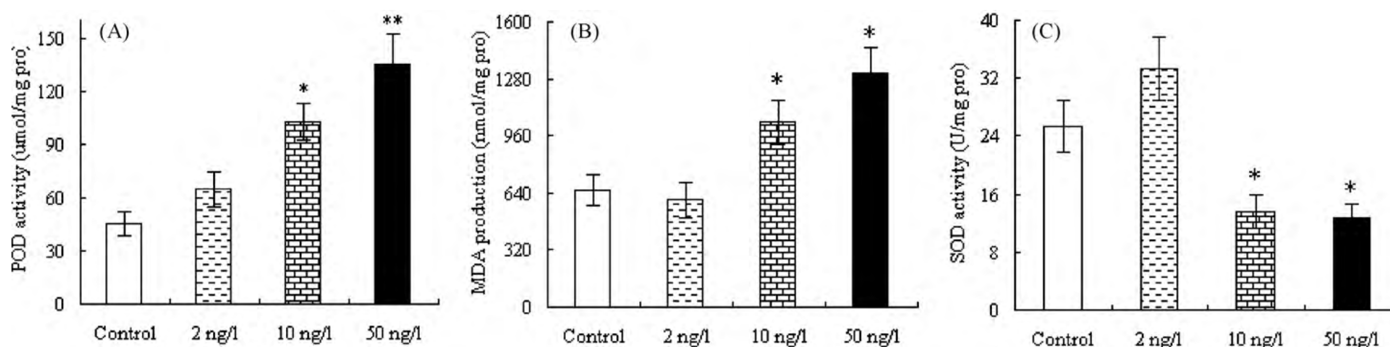

Fig. 1. The effect of TBT on antioxidant component activities in abalone. (A) POD activity, (B) MDA production, and (C) SOD level. Each bar represents the mean ± SD (n = 5). Significant differences in antioxidant component activities between the experimental and control groups are indicated with asterisks (*P < 0.05; **P < 0.01).

3. Results

3.1. Antioxidant enzyme activity

As seen in Fig. 1A, haemolymph POD activity increased in abalone after TBT exposure. The response of POD activity to TBT treatment was approximately dose-dependent, with the highest POD activity at nearly 3-fold the control (P < 0.05). Even in the lowest dose groups (2 ng/L), the POD value was increased 31.3% compared to the control, although this difference was not significant.

Dose-related MDA induction was also observed (Fig. 1B). After 2 ng/L of TBT, the MDA content was virtually the same as controls, with only a slight decrease. A 57.6% increase in MDA was observed in animals treated with 10 ng/L TBT, and a 99.5% increase was seen after 50 ng/L TBT. The trend in MDA induction was similar to the POD results, further suggesting that antioxidative balance was disturbed by TBT.

The changes in SOD activity at different TBT concentrations are shown in Fig. 1C. At the 2 ng/L dose, little difference was seen in SOD activity between the trial and controls groups, with only a slight increase in the trial groups. At higher TBT concentrations, the SOD level rapidly decreased, exhibiting a dose-dependent relationship. The lowest value was in the 50 ng/L group, which was only 0.5-fold of the control (Fig. 1C). These results showed that TBT at higher concentrations inhibited SOD activity and damaged the antioxidative competence of abalone. The reason that SOD activity in TBT-treated groups (2 ng/L) was slightly higher than in the control groups, is probably hormesis.

3.2. Metabolite analysis

Assays of abalone haemolymph metabolites after TBT exposure are in Table 1. The endogenous metabolites tested include amino acids (alanine, glutamate), organic acids (lactate, acetate, pyruvate), carbohydrates (glucose), and TCA cycle intermediates (succinate). Groups treated with 2 ng/L gave measurements that were closer to the control measurements than groups given higher doses (10 and 50 ng/L), consistent with the dose-dependent pattern (Table 1). As TBT concentrations increased, we observed a clear increase in alanine and glutamate of more than 40% in the 10 and 50 ng/L groups (P < 0.05). Exposure to TBT also resulted in a significant, dose-dependent increase in acetate level, and lactate was also markedly upregulated by TBT treatment. The highest lactate level was 1.5-fold of the control. Unlike acetate and lactate, the pyruvate level decreased significantly in TBT-treated abalones (P < 0.05). In the carbohydrates, a significant decline of haemolymph glucose was observed in abalones treated with higher doses of TBT (P < 0.05). Haemolymph glucose decreased to 35.6% in the 10 ng/L group, and 46.7% in the 50 ng/L group, compared to controls. Succinate, an intermediate of the TCA cycle, increased in both the 10 and 50 ng/L TBT groups.

3.3. Histological examination

Hepatopancreas samples from control and treated abalones were fixed, sectioned, stained and examined microscopically. No obvious histopathological changes were detected after treatment with 2 ng/L TBT. However, structural alterations, granular degeneration, and partial inflammation could be observed in samples from animals exposed to 10 and 50 ng/L (Fig. 2). Because of low differentiation in abalone organs, some guts (*e.g.* kidney and digestive glands) surrounding the hepatopancreas appeared to exhibit a partial inflammation response to TBT, although prominent morphological changes were not seen (data not shown).

Table 1
Concentration of main haemolymph metabolites in abalone exposed to different concentrations of TBT. Significant differences in metabolite levels between the experimental and the control groups are indicated with asterisks (P < 0.05).

Metabolite (μmol/mg)	Exposure groups (TBT)			
	Control	2 ng/L	10 ng/L	50 ng/L
Amino acids				
Alanine	18.54 ± 2.12	20.01 ± 2.68	25.75 ± 2.95*	27.58 ± 3.03*
Glutamine	16.95 ± 1.86	18.21 ± 2.01	18.16 ± 1.99	23.35 ± 2.46*
Organic acids				
Lactate	17.52 ± 2.15	19.34 ± 2.07	24.46 ± 1.44*	26.83 ± 3.83*
Acetate	2.74 ± 0.29	2.95 ± 0.34	3.02 ± 0.37	3.56 ± 0.43*
Pyruvate	2.68 ± 0.38	1.94 ± 0.32	1.71 ± 0.24*	1.72 ± 0.37*
Energy related				
Glucose	11.25 ± 1.56	10.46 ± 0.97*	7.33 ± 1.04*	6.10 ± 0.85*
TCA cycle intermediate				
Succinate	4.15 ± 0.54	4.09 ± 0.55	4.63 ± 0.67	4.97 ± 0.81*

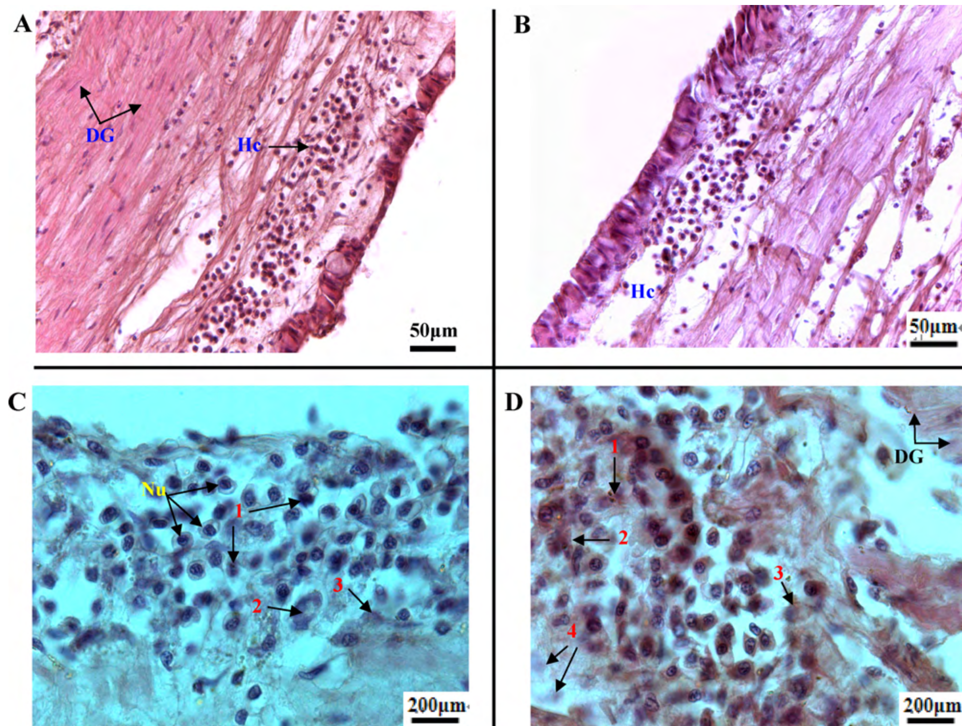

Fig. 2. Histological effects on abalone hepatopancreas after TBT exposure. (A) control; (B) 2 ng/L TBT; (C) 10 ng/L TBT and (D) 50 ng/L TBT. Signed arrows indicate the damaged positions of different tissues. Magnification: A, B 250×; C, D 1000×. Hc: hepatocytes; DG: digestion gland; Nu: nucleus; (1) nuclear alterations (granular degeneration or intracellular deposits); (2) plasma changes (cell swelling); (3) cell vacuolation; (4) infiltration or inflammation.

4. Discussion

Knowledge of antioxidant enzyme activity is extremely important for understanding the response mechanisms to stress [26]. Generally, oxidative stress refers to cellular status when the production of ROS is elevated or function of the cellular antioxidant defense system is impaired [27]. Approximately 0.1% of all oxygen entering the mitochondrial electron transport chain is released as ROS, which can disrupt intracellular redox status and result in homeostasis disorder [28,29]. Escoffier et al. [30] found that okadaic acid-induced oxidative damage disrupted the normal physiological state in medaka, and resulted in developmental malfunction. Hopkins et al. [31] reported that oxidative stress leads to teratogenesis and swim-up failure of frog larvae. Livingstone [32] demonstrated that antioxidant status, an indicator of physiological equilibrium, was critically important for understanding the response action of organisms in contaminated environment. In this work, a significant correlation was observed between the response of antioxidative components POD, MDA, and SOD to TBT exposure, which suggested that oxidative stress was induced by TBT. SOD is an important detoxification enzyme that helps prevent accumulation of oxygen radicals. We found the SOD level was reduced in abalone treated with higher doses of TBT, indicating a loss of protective capacity against cellular superoxide toxicity. MDA, a product of lipid peroxidation, is a major contributor to the loss of cell function under oxidative stress [33]. The elevation of POD reflected an increase in ROS level, while a rise in ROS may increase lipid peroxidation, as measured by MDA intensity [34]. Combining previous results with the findings of this study, suggested that oxidative damage is one of the critical toxic mechanisms of TBT. Since antioxidation and detoxification are the first line of defense to environmental stress [35], we hypothesize that abalone first initiate antioxidation and detoxification responses to counter TBT exposure. However, when antioxidation and detoxification systems break down, other toxicologically relevant responses might be induced by TBT in abalone, such as metabolic disorder and tissue damage.

Changes in metabolites involved in energy metabolism were observed. Decreased levels of glucose and pyruvate were accompanied by increased glutamate and alanine, indicating carbohydrate metabolic disturbance. Andreassen et al. [36] observed an increase in glutamate and alanine in the livers of estradiol-treated *Zoarces viviparus*, and concluded that these increases might indicate a need for high glucoplastic amino acids for energy metabolism in exposed animals. In this study, the upregulation of glutamate and alanine suggested that glycogen-derived glucose might be important for meeting the energy needs of exposed abalones. Increased lactate is also likely to be related to the energy metabolism alterations of exposed animals. An increase in hepatic lactate is often associated with an increased rate of glycolysis. Uno et al. [37] suggested that increased glycolytic activity caused the observed increase in hepatic lactate in carp after exposure to heavy oil. In addition, declining levels of pyruvate in haemolymph indicated that the trend in pyruvate metabolism was probably towards the formation of acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA stimulates pyruvate carboxylase to convert pyruvate into oxaloacetate, which can incorporate into the TCA cycle, leading to further accumulation of TCA cycle intermediates (Fig. 3). Under normal biochemical circumstances, lactate is shuttled into gluconeogenesis, and thus not maintained at low concentrations in the blood. The increase in lactate and the decrease in pyruvate suggested that TBT is a potential stimulus for gluconeogenesis.

In addition to the metabolic changes associated with energy pathways, we saw a relatively large increase in acetate after exposure of abalone to higher concentrations of TBT (10 and 50 ng/L). This indicated that TBT partially disrupted the lipid metabolism of abalone, because acetate is a product of acetyl-CoA, which is an end product of fatty acid β-oxidation (Fig. 3). The increase in acetate reflected an unbalanced lipid homeostasis induced by TBT. Moreover, the significant increase in MDA production (Fig. 1),

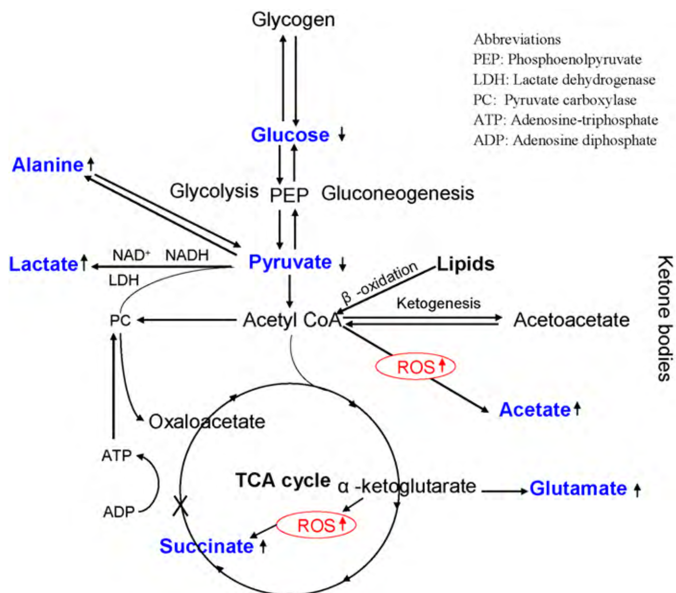

Fig. 3. Schematic representation of metabolic pathways showing the major relevant metabolic changes induced by TBT treatment in abalone haemolymph. Up arrows indicate increased concentrations, and down arrows indicate decreased concentrations after TBT. In TBT conditions, carbohydrate metabolism, lipid metabolism and TCA cycle were affected, which could disrupt physiological status. The conversion of pyruvate to acetate and α-ketoglutarate to succinate is accompanied by aerobic respiration, which would indirectly increase ROS levels and cause oxidative stress.

which indicates lipid peroxidation status, also indicated that TBT disrupted lipid metabolism in abalone. Belpaire and Goemans [38] reported that contaminants perturbed the lipid metabolism of eel, and concluded that changes in lipid peroxidation levels might be partly responsible for impairment in growth and/or reproduction. Ding et al. [14] demonstrated that hepatotoxicity resulted in alterations in lipid metabolism of rat after chronic Perfluorooctanoic acid exposure. In gastropod mollusks, the hepatopancreas is an important multifunctional organ, controlling immunity, antioxidation, and lipid synthesis or storage. In this study, histopathological changes in the hepatopancreas further suggested that TBT treatment perturbed lipid metabolism in abalone [39].

Effects on amino acid metabolism were partially supported by an increase in a TCA cycle intermediate, succinate. This increase in haemolymph suggested anaerobic respiration, and slowing of the TCA cycle and electron transport chain. Succinate represents a connection between the TCA cycle and the electron transport chain. Under normoxic conditions, succinate is oxidized to fumarate by succinate dehydrogenase, and electrons are transferred to FAD to form $FADH_2$, in the electron transport chain. If the electron transport chain is slowed, then regeneration of FAD from $FADH_2$ decreases and succinate oxidation also decreases leading to glutamate accumulation, as observed here (Fig. 3). The deceleration of the TCA cycle causes other TCA cycle intermediates to accumulate, for example acetyl-CoA, which breaks down and releases acetate, as we observed (Fig. 3). In addition, upregulation of succinate, to some extent, reflects ROS increase and the conversion of α-ketoglutarate to succinate is accompanied by aerobic respiration, which would indirectly increase ROS levels, leading to oxidative stress [40] (Figs. 1 and 3).

5. Conclusion

We demonstrated that TBT induced oxidative stress and disturbed the antioxidant balance in abalone by inhibiting SOD activity and POD levels, and increasing MDA production. Histopathological observations showed that TBT might injure the involved functional organ directly or indirectly. More importantly, TBT disturbed the physiological status of abalone, including energy, lipid, and amino acid metabolism. These results suggested that TBT may have multiple potential toxic effects on abalone. This work will improve our understanding of the toxicological mechanisms of TBT, and provide guidance for deeper investigation, for example for metabolomics studies.

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