



## Evaluation of mitochondrial toxicity of cadmium in clam *Ruditapes philippinarum* using iTRAQ-based proteomics<sup>★</sup>

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### ABSTRACT

Cadmium is one of the most serious metal pollutants in the Bohai Sea. Previous studies revealed that mitochondrion might be the target organelle of Cd toxicity. However, there is a lack of a global view on the mitochondrial responses in marine animals to Cd. In this work, the mitochondrial responses were characterized in clams *Ruditapes philippinarum* treated with two concentrations (5 and 50 µg/L) of Cd for 5 weeks using tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining, ultrastructural observation and quantitative proteomic analysis. Basically, a significant decrease of mitochondrial membrane potential ( $\Delta\Psi_m$ ) was observed in clams treated with the high concentration (50 µg/L) of Cd. Cd treatments also induced specific morphological changes indicated by elongated mitochondria. Furthermore, iTRAQ-based mitochondrial proteomics showed that a total of 97 proteins were significantly altered in response to Cd treatment. These proteins were closely associated with multiple biological processes in mitochondria, including tricarboxylic acid (TCA) cycle, oxidative phosphorylation, fatty acid  $\beta$ -oxidation, stress resistance and apoptosis, and mitochondrial fission. These findings confirmed that mitochondrion was one of the key targets of Cd toxicity. Moreover, dynamical regulations, such as reconstruction of energy homeostasis, induction of stress resistance and apoptosis, and morphological alterations, in mitochondria might play essential roles in Cd tolerance. Overall, this work provided a deep insight into the mitochondrial toxicity of Cd in clams based on a global mitochondrial proteomic analysis.

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### 1. Introduction

With the rapid development of industry, cadmium (Cd) has become one of the most serious metal pollutants in the Bohai Sea because of a huge amount of Cd discharge from industrial effluents (Ji et al., 2016; Luo et al., 2013). Many surveys, including those by our group, have demonstrated Cd pollution in multiple environmental media in the Bohai Sea (Gao et al., 2014; Ji et al., 2016; Xu et al., 2016a). Based on the published data, the seawater Cd

concentration has reached to 5 µg/L in the Jinzhou Bay and 16 µg/L in the Liaodong Bay in the Bohai Sea, respectively (Ma et al., 1995, Zhang et al., 2001). Moreover, our findings showed that Cd was one of the major metal pollutants accumulated in shrimp *Crangon affinis* that is a representative benthic species in the Bohai Sea (Xu et al., 2016a). These evidences confirm the historic contamination of Cd in the Bohai Sea, and the threats of Cd to marine organisms certainly deserve to be further concerned.

Increasing studies have been conducted on the threats of Cd to marine organisms, demonstrating that Cd could inhibit growth, disrupt energy metabolism, and induce immune and oxidative stress in marine organisms (Fernandez-Leborans and Herrero, 1999; Ji et al., 2015a; Qu et al., 2013; Sokolova, 2004; Wang and Rainbow, 2006; Wu et al., 2017). Especially, our previous work showed that Cd could affect energy metabolism in marine bivalves, indicated by the alterations of metabolites including glucose, amino

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acids, ATP, ADP, and other intermediates in tricarboxylic acid (TCA) cycle (Ji et al., 2015a; Wu et al., 2017). The mechanisms of Cd toxicity have been widely but fully explored. Based on so-called ionic mimicry, Cd exerts its toxic effects by replacing elements like calcium ( $\text{Ca}^{2+}$ ) and trace elements like zinc ( $\text{Zn}^{2+}$ ) (Choong et al., 2014). The overproduction of reactive oxygen species (ROS) was also reported to be induced by Cd via binding to the thiol groups of proteins, subsequently leading to oxidative stress, DNA damage, lipid peroxidation, and even apoptosis (Chen et al., 2015; Gobe and Crane, 2010). Interestingly, all these evidences suggested that mitochondrion should be the target organelle of Cd toxicity.

As it is known, mitochondria are dynamic organelles responsible for ATP production through oxidative phosphorylation (OXPHOS) and play an essential role in a diverse array of metabolic processes, such as apoptosis, calcium h cycle, fatty acid metabolism, amino acid metabolism, and heme biosynthesis (Meyer et al., 2013). Considering the complexity of mitochondria, it is not surprising that they are often deemed as the target of pollutant-induced toxicity (Meyer et al., 2013). The mitochondrial toxicity of Cd has been reflected in influencing mitochondrial bioenergetics and gene expressions of key mitochondrial proteins, as well as causing oxidative stress (Gobe and Crane, 2010; Sokolova et al., 2005). For example, Sokolova et al. (2005) observed that Cd can be accumulated in mitochondria of oyster, which subsequently led to impaired capacity for ATP production. Though the toxicological effects of Cd to mitochondria have been confirmed, there is an obvious lack of global characterization of mitochondrial responses to Cd. Hence, a comprehensive evaluation of mitochondrial responses is vital to further understanding the mitochondrial toxicity of Cd.

Intertidal organisms, including mollusks, crustaceans and fishes, are vulnerable to environmental insults and mainly rely on mitochondria to adapt exogenous stresses (Sokolova, 2018). Clam *Ruditapes philippinarum* is a typical intertidal species in the Bohai Sea, which has been frequently used as a preferable indicator in pollution biomonitoring because of its wide distribution and high tolerance to pollutants (Ji et al., 2015b; Liu et al., 2011; Marques et al., 2018). Proteomics that focuses on the global changes of proteome has been frequently used to characterize the toxicological mechanisms of pollutants. Our previous work using proteomics approach revealed that Cd could disturb energy metabolism and induce oxidative stress in bivalves, implying that mitochondrion might be the target of Cd (Xu et al., 2016b). Considering the importance of mitochondria and its susceptibilities to Cd, it is necessary to characterize the global response of mitochondria to Cd at protein level. Mitochondrial proteomics is capable of providing new insights into mitochondrial function, and has been increasingly used in studies on human disease and ageing (Calvo and Mootha, 2010). To our knowledge, few studies have focused on the global characterization, at protein level, of mitochondrial responses of clams to Cd treatment. In the present study, high-throughput proteome screening based on iTRAQ technique was conducted to detect the responsive proteins in clam mitochondria, aiming to unravel the strategy of mitochondrial responses of clams to Cd treatment.

## 2. Materials and methods

### 2.1. Animals and experimental design

Adult clams *R. philippinarum* (shell length: 3.5–3.8 cm, White pedigree) were purchased from a culturing farm (Qingdao, China) and acclimated in aerated seawater in laboratory for 7 days. After acclimation, animals were then randomly divided into three groups (control group and two Cd-treated groups). The clams cultured in the normal filtered seawater were used as control group. Other two

groups of clams were exposed to two sublethal concentrations (5 and 50  $\mu\text{g/L}$ ) of Cd by the addition of stock solutions of  $\text{CdCl}_2$ , which were prepared using deionized water and analytical reagent grade compounds. Each treatment consisted of 30 individuals and was performed in triplicate per group. Seawater was completely replaced by fresh seawater every day. During the acclimation and exposure periods, clams were kept in aerated seawater ( $25 \pm 2^\circ\text{C}$ , 31 psu) under a photoperiod of 12 h light and 12 h dark, fed with *Chlorella vulgaris* of 2% tissue dry weight once daily. After exposure for 35 days, gill tissues of clams were immediately dissected for subsequent analyses.

### 2.2. Isolation of mitochondria

Mitochondria were isolated from gills according to (Li et al., 2009) with some modifications. To keep the mitochondria alive, all steps were performed at  $4^\circ\text{C}$ . In brief, the fresh gill tissues were transferred to pre-cooled tubes, followed by Dounce homogenization (20 strokes) in lysis buffer I (LB I: 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4) containing protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The homogenate was centrifuged at 1500 g for 10 min, and the supernatant was reserved. The pellet was re-suspended in lysis buffer and the centrifugation at 1500 g for 10 min was repeated twice more. The supernatants were combined and treated with an additional centrifugation at 10,000 g for 10 min. The pellet containing crude mitochondria were re-suspended and stored in LB I. Further enrichment was conducted on the crude mitochondria by centrifugation at a serial of Nycodenz<sup>®</sup> gradients at 40,000 g for 45 min. The gradient solutions (0.5 mL of 20%, 2 mL of 23%, 2.5 mL of crude mitochondria in 25%, 2 mL of 30% and 1.2 mL of 34 and 40%) were prepared from 80% Nycodenz<sup>®</sup> stock (10 mM HEPES, 1 mM EGTA, pH 7.4). The pure mitochondria fraction with a pale yellow color between 25 and 30% gradients was carefully collected and washed with LB I, and then stored in lysis buffer II (9 M urea, 4% CHAPS, 1% w/v DTT) for further proteomic analysis.

### 2.3. Mitochondrial morphology by transmission electron microscopy

Transmission electron microscopy (TEM) of gill mitochondria was carried out to evaluate the morphological alterations of mitochondria in gills between control and Cd-treated clams. Detailed descriptions on production and examination of sections were available in the Supporting Information S1.

### 2.4. Measurement of mitochondrial membrane potential

The assessment of mitochondrial membrane potential (MMP,  $\Delta\Psi_m$ ) was conducted using a commercial kit (Beyotime, Nantong, China) based on the mitochondrial-specific fluorescent probe tetraethylbenzimidazolylcarbocyanine iodide (JC-1). In mitochondria with high  $\Delta\Psi_m$ , JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence (590 nm). Oppositely, JC-1 remains in monomeric form in mitochondria with low  $\Delta\Psi_m$ , presenting green fluorescence (527 nm). In brief, each crude mitochondria sample isolated from gills of 9 clams was mixed into JC-1 reagent with the volume ratio of 9 to 1. The mixtures were detected immediately using BD FACSAria flow cytometer (Becton, NJ, USA) at 490 nm excitation. JC-1 monomers and aggregates showing green and red spectral shifts were measured in FL1 channel (515–545 nm) and FL2 channel (562–588 nm), respectively. Data were then processed using FACSAria Diva software 6.1.3 and plotted on histogram of FL2 versus FL1, and the ratio of FL2/FL1 for JC-1 is generally used to indicate  $\Delta\Psi_m$  in stained mitochondria.

The ratio was shown as mean  $\pm$  standard deviation ( $n = 4$ ). One way analysis of variance (ANOVA) was conducted to evaluate the difference of ratios between control and Cd treatment groups, and a  $p$  value less than 0.05 was considered statistically significant.

## 2.5. Proteomic analysis

Each pure mitochondria sample isolated from gills of 13 clams was dissolved in lysis buffer (9 M urea, 4% CHAPS, 1% w/v DTT) with protease inhibitor (Roche Applied Science, Mannheim, Germany). The concentrations of the protein extracts were determined using the Bradford method (Bradford, 1976). iTRAQ technique was applied for quantitative proteomic analysis. Briefly, 100  $\mu$ g of protein extracted from each sample was dissolved in the dissolution buffer (AB SCIEX, MA, USA). After being reduced, alkylated and trypsin-digested, the protein samples were labeled using iTRAQ 4-plex reagents (AB SCIEX, MA, USA) according to the manufacturer's protocol. Samples from control, low concentration (5  $\mu$ g/L) of Cd treatment, and high concentration (50  $\mu$ g/L) of Cd treatment groups were labeled with 119-, 117-, and 118-tag, respectively. A pooled sample from three groups was labeled with 121-tag for internal normalization. Three biological replicates were used in each group. The peptides were then purified from excess labeling reagent by strong cation exchange (SCX) chromatography using Agilent 1200 HPLC (Agilent, CA, USA), followed by being separated using Eksigent nanoLC-Ultra 2D system (Eksigent, CA, USA). The LC fractions were analyzed by MS/MS using a Triple TOF 5600 mass spectrometer (AB SCIEX, MA, USA).

Protein identification and relative quantification were performed with ProteinPilot Software 5.0 (AB SCIEX). MS/MS data were searched against a mollusk protein database (translated from *R. philippinarum* transcriptome database with 38,224 sequences, built in 2013) and a decoy database for false discovery rate (FDR) analysis. The database search parameters were the followings: iTRAQ 4-plex quantification, cysteine modified with iodoacetamide, methionine modifications of oxidation, trypsin digestion, one missed cleavage allowed, peptide tolerance was set as 0.05 Da, and MS/MS tolerance was set as 0.05 Da. Only peptides identified with local FDR <0.05 were considered for further analysis. Proteins with unused score >1.3, matching reliable peptides ( $\geq 2$  unique peptides) were considered as positively identified proteins. Protein ratio was calculated by averaging the ratios of three replicates, and protein with a fold change ratio >1.2 or <0.83 as well as a  $p$  value <0.05 in three replicates was considered as differentially expressed proteins (DEPs). Evaluation of function of DEPs was performed according to NCBI (<https://www.ncbi.nlm.nih.gov/>), Uniprot (<https://www.uniprot.org/>) and KEGG (<https://www.genome.jp/kegg/>) database analyses. More detailed information of iTRAQ analysis, protein identification and quantification was described in the Supporting Information S1.

## 2.6. Nuclear magnetic resonance-based metabolomic analysis

Nuclear magnetic resonance (NMR)-based metabolomics was used to reveal the alterations of metabolites in gill samples of clams treated with Cd. Detailed information was available in Supporting Information S1.

## 3. Results and discussion

### 3.1. Mitochondrial membrane potentials ( $\Delta\Psi_m$ )

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) is primarily generated by proton pumps (Complex I, III, IV) and essential for ATP production (Zorova et al., 2018). It also provides a driving force for

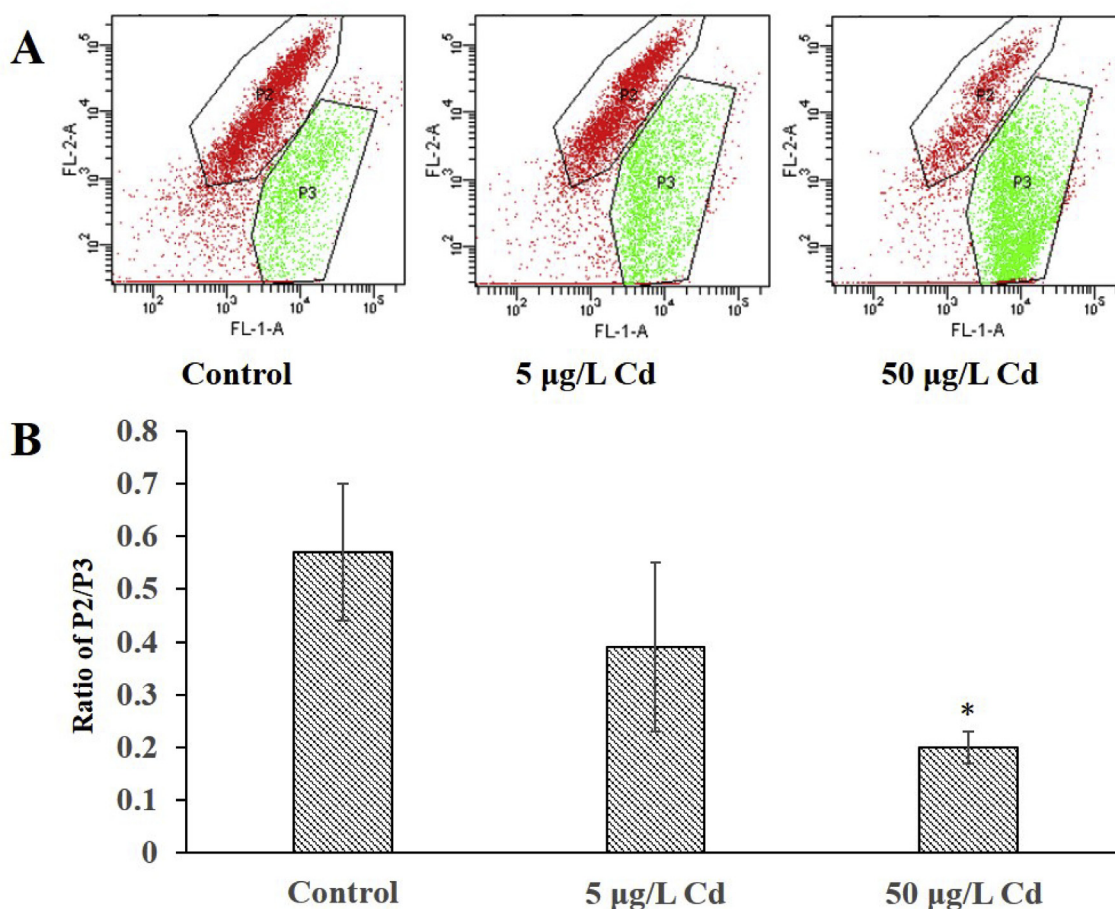
transport of ions (except for  $H^+$ ) and proteins which are necessary for mitochondrial function (Zorova et al., 2018). The measurement of  $\Delta\Psi_m$  based on JC-1 probe has been reported as a suitable candidate for assessing pollution toxicity in fish (Padmini and Rani, 2011). As shown in Fig. 1A, partitions of P2 (red) and P3 (green) represented mitochondria with high and low  $\Delta\Psi_m$ , respectively. The ratio of P2/P3 was used to indicate  $\Delta\Psi_m$ . The results demonstrated that both concentrations of Cd caused decreases of  $\Delta\Psi_m$ . However, the significant ( $p < 0.05$ ) alteration of  $\Delta\Psi_m$  was uniquely found in the high concentration (50  $\mu$ g/L) of Cd-treated clams. Cd was prone to attach to the inner membrane of mitochondria and enhance the membrane permeability, resulting in  $H^+$  efflux (Muller, 1986; Rasheed et al., 1984). In fact, the negative effect of Cd to  $\Delta\Psi_m$  has been frequently reported in cells or isolated mitochondria (Belyaeva et al., 2008; Li et al., 2003). Oyster mitochondria treated with Cd presented a significant decrease of ATP synthesis, which was partially attributed to mitochondrial membrane potential dissipation (Sokolova et al., 2005). Consistently, the decreases of  $\Delta\Psi_m$  were also observed in clams treated with Cd in our case, especially in the high concentration (50  $\mu$ g/L) of Cd-exposed individuals, indicating that Cd possibly influenced ATP synthesis and healthy function of mitochondria.

### 3.2. Global mitochondrial proteome alterations in response to Cd treatment

Analyses of three biological replicates resulted in 864, 976 and 930 proteins, respectively (Supporting Information S2). A total of 383 proteins were identified in all three replicates, among which 97 proteins were differentially expressed in response to Cd treatments. Considering that many DEPs were hypothetical proteins with unknown functions, further blast was conducted on DEPs by searching database of Non-redundant protein sequences database (nr) using blastp, resulting in a total of 26 out of 97 DEPs identified as known mitochondrial proteins. In order to characterize the mitochondrial responses of clams to Cd treatment, we only focused on function analysis of mitochondrial DEPs, which were listed in Table 1. Detailedly, among these 26 DEPs, 21 and 16 DEPs were detected in clams treated with 5  $\mu$ g/L and 50  $\mu$ g/L Cd, respectively. In the low concentration (5  $\mu$ g/L) of Cd-exposed group, there were 6 up-regulated and 15 down-regulated DEPs. Three up-regulated and thirteen down-regulated DEPs were identified in clams treated with the high concentration (50  $\mu$ g/L) of Cd. It is noteworthy that only 9 out of 26 DEPs exhibited common alteration tendency in both Cd-treated groups. The differential proteomic responses implied that there might be dose-dependent effects of Cd on clam mitochondria.

### 3.3. Mitochondrial morphology

Mitochondrial morphologies have been reported to be often associated with energetic states and survival of cells (Li et al., 2017). Normally, active mitochondria continually alter its shapes, such as fission and fusion, to maintain mitochondrial functions when cells experience metabolic or environmental stresses (Youle and van der Bliek, 2012). In this work, TEM was conducted to characterize the influences of Cd on the ultrastructure of mitochondria. Representative mitochondrial elongations were observed in clams from both Cd-treated groups (Fig. 2). Interestingly, proteomic analysis demonstrated that several proteins related to regulating mitochondrial morphology were also significantly altered in Cd-exposed clams. Dynamin-1 like protein (DLP1) shares up to 43% homology with dynamin-related protein 1 (DRP1) which plays a critical role in the mitochondrial fission (Kanamaru et al., 2012; Yoon et al., 1998). Evidences have confirmed that down-regulation



**Fig. 1.** Impact of Cd on mitochondrial membrane potential ( $\Delta\Psi_m$ ) in control and Cd-treated clams indicated by JC-1 staining. The change in mitochondrial membrane potential ( $\Delta\Psi_m$ ) was expressed as the ratio of mitochondria with high  $\Delta\Psi_m$  to those with low  $\Delta\Psi_m$ . **A:** Representative FACS blots of mitochondria stained by JC-1. Partitions P2 (red) and P3 (green) present mitochondria with high  $\Delta\Psi_m$  and low  $\Delta\Psi_m$ , respectively. **B:** The bar graphs indicate the relative ratios of P2/P3, which are presented as the mean  $\pm$  standard deviation. Statistical significances ( $p < 0.05$ , \*) between control and Cd treatments were determined by one way ANOVA.

or inhibition of DRP1 contributed to mitochondrial elongation and swelling by regulating calcium homeostasis (Frank et al., 2001; Mai et al., 2010; Xu et al., 2013; Yu et al., 2016). In this work, the down-regulation of DRP1 were highly coincident with mitochondrial elongation in Cd-treated clams. Apart from catalyzing the synthesis of glutamate from proline, delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH) is also required for mitochondrial integrity. The loss of P5CDH was associated with mitochondrial swelling (He and DiMario, 2011). Moreover, CDGSH iron-sulfur domain-containing protein 2 (CISD2) is well known as a regulator of autophagy, and its abnormal regulation was always linked to environmental stimulus like ocean acidification (Timmins-Schiffman et al., 2014). In this work, P5CDH was significantly down-regulated in clams from both Cd treatments, while decreases of CISD2 and DLP1 were only observed in 5 μg/L and 50 μg/L Cd-treated clams, respectively. The alterations at protein and ultrastructure levels suggested that mitochondrial morphology was altered to adapt Cd exposure.

### 3.4. Energy metabolism

#### 3.4.1. TCA cycle

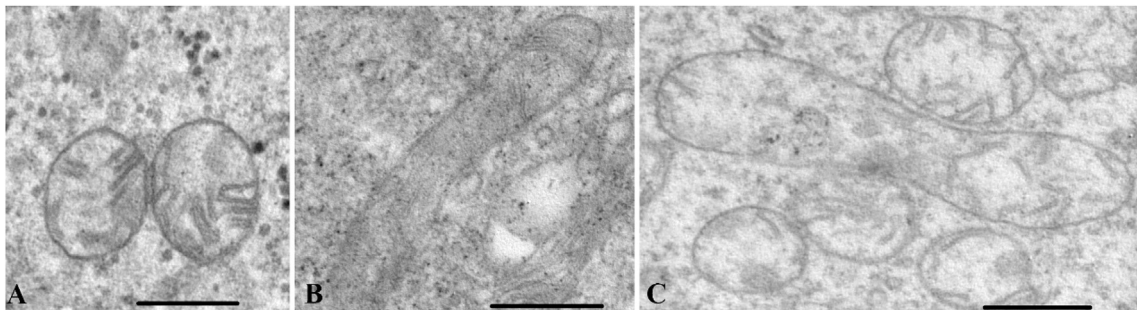
TCA cycle, also named as citric acid cycle or Krebs cycle, is a central hub of carbon metabolism coordinating the metabolism of glucose, amino acids, respiration, and biosynthetic pathways. It generates co-enzymes like NADH and FADH<sub>2</sub> used in electron transport chain (ETC). In this work, five proteins related to TCA

cycle, including NAD(P) transhydrogenase (nicotinamide nucleotide transhydrogenase, NNT), mitochondrial glutamate dehydrogenase 1 (GLUD1), phosphoenolpyruvate carboxykinase (PEPCK), propionyl-CoA carboxylase alpha (PCCA), and mitochondrial dihydroliipoamide dehydrogenase (DLD), were inhibited by Cd treatments. Among these proteins, NNT, GLUD1 and PEPCK were down-regulated in both Cd treatments, while other two proteins, PCCA and DLD, were decreased in the high concentration (50 μg/L) of Cd-treated group. NNT catalyzes the transfer of hydride ion equivalents from NADH to NADPH using the proton gradient, whose knock-down inhibited the contribution of glutamine to the TCA cycle and activates glucose catabolism (Gameiro et al., 2013). In this work, Cd treatments caused significant ( $p < 0.05$ ) down-regulations of NNT in clams, which might subsequently weaken glutamine utilization and enhance glucose utilization in TCA cycle. GLUD1 catalyzes the reversible conversion of glutamate to  $\alpha$ -ketoglutarate. The down-regulated GLUD1 caused by Cd treatments implied the alleviation of  $\alpha$ -ketoglutarate supply to TCA cycle. PEPCK functions as a hub molecule that links TCA cycle intermediates and glycolytic pools through the conversion of mitochondrial oxaloacetate into phosphoenolpyruvate (Stark et al., 2014). The expression of PEPCK has been reported to be negatively regulated by glucose (Vincent et al., 2015). Interestingly, all these proteins, including NNT, GLUD1, and PEPCK, are associated with regulation of glucose and (or) glutamine. Normally, both glucose and glutamine can be involved in TCA cycle by providing intermediates such as acetyl-CoA and  $\alpha$ -

**Table 1**  
The list of differentially expressed proteins in gill mitochondria from clams treated with Cd.

Accession	E value	Uniprot	Gene name	Description	FC 5 <sup>a</sup>	FC 50 <sup>b</sup>
<b>TCA cycle</b>						
c130046_g1	0	P00367	GLUD1	Glutamate dehydrogenase 1, mitochondrial	0.64	0.77
c126333_g1	0	Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	0.38	0.50
c120997_g1	0	Q13423	NNT	NAD(P) transhydrogenase, mitochondrial	0.61	0.49
c131875_g1	0	P05165	PCCA	Propionyl-CoA carboxylase alpha chain, mitochondrial		0.51
c124115_g1	0	P09622	DLD	Dihydrolipoyl dehydrogenase, mitochondrial		0.44
<b>Oxidative phosphorylation</b>						
c112081_g1	2.00E-15	Q9Y6M9	NDUFB9	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	0.69	
c122165_g1	8.00E-103	O75489	NDUFS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	1.68	1.32
c115143_g1	1.00E-90	P47985	UQCRC1	Cytochrome <i>b-c1</i> complex subunit 11	0.53	
c125555_g1	5.00E-19	P22695	UQCRC2	Cytochrome <i>b-c1</i> complex subunit 2, mitochondrial	0.58	
c114452_g1	3.00E-21	P20674	COX5A	Cytochrome <i>c</i> oxidase subunit 5A, mitochondrial	2.33	0.73
c115262_g1	1.00E-35	P24539	ATP5F1	ATP synthase F(0) complex subunit B1, mitochondrial	1.41	0.75
c115113_g1	1.00E-31	P30049	ATP5D	ATP synthase subunit delta, mitochondrial		0.36
c119322_g1	4.00E-89	Q9HAN9	NMNAT1	Nicotinamide/nicotinic acid mononucleotide adenyltransferase 1	0.61	0.47
c78903_g1	4.00E-45	Q9UIJ7	AK3	GTP:AMP phosphotransferase AK3, mitochondrial	0.42	
c133792_g1	0	P43304	GPD2	Glycerol-3-phosphate dehydrogenase, mitochondrial	0.77	
<b>Morphology</b>						
c131435_g1	0	P30038	ALDH4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase	0.41	0.47
c122087_g1	7.00E-22	Q8N5K1	CISD2	CDGSH iron-sulfur domain-containing protein 2	0.35	
c135035_g1	1.00E-25	O00429	DNM1L	Dynamin-1-like protein		0.74
<b>Fatty acid <math>\beta</math>-oxidation</b>						
c130064_g1	3.00E-176	P42765	ACAA2	3-ketoacyl-CoA thiolase, mitochondrial		0.70
c134067_g1	0	P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial	0.61	0.54
<b>Stress resistance and apoptosis</b>						
c133979_g1	9.00E-154	Q9NRK6	ABC10	ATP-binding cassette sub-family B member 10, mitochondrial	1.32	1.20
c129163_g1	0	P05091	ALDH2	Aldehyde dehydrogenase, mitochondrial	1.45	
c131764_g1	9.00E-146	Q9Y6N5	SQRDL	Sulfide:quinone oxidoreductase, mitochondrial	0.57	
c133597_g1	0	O00571	DDX3X	ATP-dependent RNA helicase DDX3X	0.43	
c124602_g1	3.00E-138	Q9UJZ1	STOML2	Stomatin-like protein 2, mitochondrial	1.54	1.45
c112823_g1	4.00E-138	P45880	VDAC2	Voltage-dependent anion-selective channel protein 2	0.48	

<sup>a, b</sup> the fold changes of DEPs in the low (5  $\mu$ g/L) and high (50  $\mu$ g/L) concentrations of Cd-treated groups, respectively, compared to those in control ( $p < 0.05$ ).



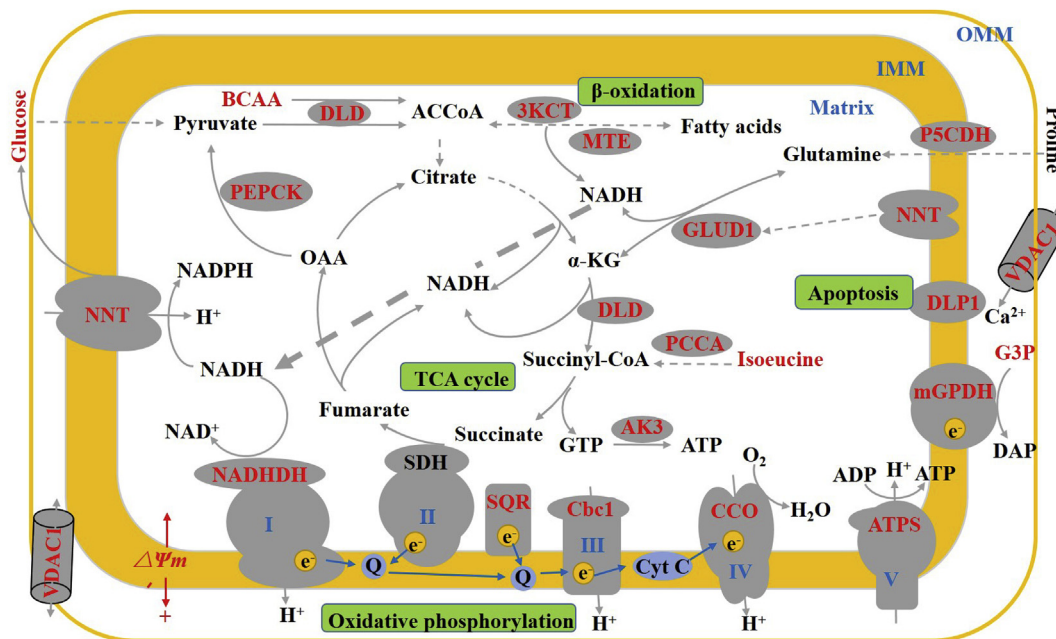
**Fig. 2.** TEM micrographs of mitochondria from clam gills. **A:** Control group. **B:** 5  $\mu$ g/L Cd treatment group. **C:** 50  $\mu$ g/L Cd treatment group. Scale bar: 500 nm.

ketoglutarate. In some cases, glucose and glutamine compensate for each other to maintain TCA cycle function (Yang et al., 2014). In this work, the down-regulations of NNT, GLUD1 and PEPCK were consistently observed in clams treated with Cd, indicating that the pathway linked to TCA cycle via glutamine was alleviated while the pathway via glucose was enhanced. Interestingly, the alterations of these proteins were highly consistent with the increased glucose in Cd-treated clams characterized by metabolomic analysis (Fig. 3, Supporting Information S1, Fig. 1S), confirming the increased demand of glucose for TCA cycle. It is noteworthy that two more proteins, PCCA and DLD, were down-regulated in clams from 50  $\mu$ g/L Cd-treated group. PCCA is linked to producing methylmalonyl-CoA which is then converted into succinyl-CoA, an intermediate in the TCA cycle (Wongkittichote et al., 2017). DLD is regarded as a critical component of multiple mitochondrial dehydrogenase complexes including pyruvate dehydrogenase complex,  $\alpha$ -ketoglutarate dehydrogenase complex, and branched chain amino acid (BCAA) dehydrogenase complex. It provides succinyl-CoA and

acetyl-CoA to TCA cycle. The down-regulation of PCCA and DLD signified that the high concentration (50  $\mu$ g/L) of Cd might impede the influxes of succinyl-CoA and acetyl-CoA into TCA cycle. That is to say, the high concentration of Cd (50  $\mu$ g/L) induced more obvious inhibition of TCA cycle than the low concentration (5  $\mu$ g/L) of Cd in clam mitochondria. Overall, the proteomic responses in TCA cycle indicated that mitochondria had to adapt Cd stimulus by changing its strategy of energy supply.

#### 3.4.2. Oxidative phosphorylation (OXPHOS)

Taking place inside mitochondria, oxidative phosphorylation (OXPHOS) provides most of the energy needed for cellular functions. OXPHOS mainly consists of 5 complexes that belong to two sets of reactions, electron transport chain (ETC) and ATP synthesis. In this work, four out of five complexes in OXPHOS, including NADH dehydrogenase [ubiquinone] (complex I), cytochrome *bc1* (complex III), cytochrome *c* oxidative (complex IV) and ATP synthase (complex V), were significantly altered in Cd-treated clam



**Fig. 3.** A schematic presentation of global responses of mitochondria from clam gills exposed to Cd treatment. The proteins, metabolites, as well as other intermediates that were involved in TCA cycle, oxidative phosphorylation,  $\beta$ -oxidation and apoptosis were included in the schematic, and the responsive proteins and metabolites were marked in red. Abbreviation:  $\alpha$ -KG,  $\alpha$ -ketoglutarate; 3KCT, 3-ketoacyl-CoA thiolase; ACCoA, acetyl-CoA; AK3, GTP:AMP phosphotransferase; ATPS, ATP synthesis; BCAA, branched chain amino acid; Cbc1, cytochrome bc1; CCO, cytochrome c oxidase; DLD, dihydrolipoamide dehydrogenase; DLP1, dynamin-1 like protein; G3P, glycerol-3-phosphate; GLUD1, glutamate dehydrogenase 1; IMM, inner mitochondrial membrane; mGPDH, glycerol-3-phosphate dehydrogenase; MTE, mitochondrial trifunctional enzyme; NADHDH, NADH dehydrogenase; NNT, nicotinamide nucleotide transhydrogenase; OAA, oxaloacetate; OMM, outer mitochondrial membrane; P5CDH, delta-1-pyrroline-5-carboxylate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; SDH, succinate dehydrogenase; SQR, sulfide quinone oxidoreductase; VDAC1, voltage-dependent anion-selective channel 1.

mitochondria. As the first and largest of complexes of ETC, complex I catalyzes NADH that are generated from TCA cycle and  $\beta$ -actin into  $\text{NAD}^+$ . In this work, two subunits of complex I were differentially expressed with different tendencies. NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 is regarded as an accessory subunit of complex I and not involved in catalysis, whereas NADH dehydrogenase [ubiquinone] iron-sulfur protein 3 is one of the core subunits of complex I and belongs to minimal assembly required for catalysis (Murray et al., 2003; Stroud et al., 2016). Complex III was ever regarded as the only site in the ETC complex where ROS were produced in the presence of Cd (Wang et al., 2004). Complex IV catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen. Complex V produces ATP from ADP in the presence of a proton gradient (MMP) across the membrane which is generated by electron transport complexes of the respiratory chain. In the low concentration (5  $\mu\text{g/L}$ ) of Cd-treated group, six proteins involved in complex I, III, IV and V were altered with inconsistent tendencies, which were even observed in two subunits of one complex. Differing from the low concentration (5  $\mu\text{g/L}$ ) of Cd-treated group, four proteins related to complex I, IV and V were differentially expressed in the high concentration (50  $\mu\text{g/L}$ ) of Cd-treated group. Previous evidences confirmed the toxicological effects of Cd to OXPHOS (Cannino et al., 2009; David et al., 2010; Izrael-Zivkovic et al., 2018; Sokolova et al., 2005; Wang et al., 2004). Especially, Sokolova et al. (2005) observed that Cd exposure significantly decreased ADP-stimulated respiration (state 3), leading to impaired capacity of ATP production. However, some studies have given results differing from what were found in this work. Wang et al. (2004) reported that complex II and III of ETC from pig liver, brain and heart presented greater inhibition by Cd than the other complex. These inconsistent results of responses to Cd might suggest the differential mechanisms of Cd toxicity between marine clams and mammals. Furthermore, some proteins indirectly related

to OXPHOS were also found to be differentially expressed in responses to Cd treatments. As a cofactor, NAD is essential in connecting TCA cycle and respiratory chain. Nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) is the master enzyme of NAD biosynthesis in living organisms (Zhai et al., 2009). The down-regulation of NMNAT in both Cd treatments indicated that Cd inhibited the NAD biosynthesis in mitochondria. GTP:AMP phosphotransferase (AK3) contributes to the interconversion of adenine nucleotides and plays an important role in cellular energy homeostasis (Muller et al., 2017). Mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) is a flavin-linked respiratory chain dehydrogenase that oxidizes glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DAP), and serves as an important enzyme of intermediary metabolism that links glycolysis, oxidative phosphorylation and fatty acid metabolism (Mracek et al., 2013). The down-regulated GPDH in 5  $\mu\text{g/L}$  Cd-treated group was consistent with the elevated G3P that was characterized by metabolomic analysis (Fig. 3, Supporting Information S1, Fig. 1S). Down-regulations of these two proteins, including AK3 and GPDH, induced by 5  $\mu\text{g/L}$  Cd confirmed that energy homeostasis were reconstructed in Cd-treated clams.

### 3.4.3. Fatty acid $\beta$ -oxidation

Fatty acid  $\beta$ -oxidation (FAO) is an important catabolic process by which fatty acids are broken down to generate acetyl-CoA, NADH and  $\text{FADH}_2$ . Then acetyl-CoA enters the TCA cycle, and NADH and  $\text{FADH}_2$  were used in electron transport chain (ETC) (Wang et al., 2010). Mitochondrial trifunctional enzyme (MTE), also known as hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, is an  $\alpha_2\beta_2$  complex consisting of two types of polypeptides catalyzing three of the four reactions of the  $\beta$ -oxidation (Venkatesan and Wierenga, 2013). 3-ketoacyl-CoA thiolase (3KCT) functions as an enzyme catalyzing the last step of the

mitochondrial  $\beta$ -oxidation pathway (Uchida et al., 1992). In this work, MTE were decreased in both Cd treatment groups, while reduction of 3KCT were observed in the high concentration (50  $\mu\text{g/L}$ ) of Cd-treated group only. The down-regulations of MTE and 3KCT indicated an inhibition of FAO in Cd-treated clams, subsequently leading to decreased supply of acetyl-CoA and NADH to TCA cycle and OXPHOS, respectively.

### 3.5. Stress resistance and apoptosis

As energy powerhouses, mitochondria are also helpful in resistance to stress in organisms especially in intertidal bivalves (Sokolova, 2018). Mitochondrial stress like over-production of ROS not only impedes ATP synthesis via influencing mitochondrial membrane potential and ETC, but also elevates steady-state cytosolic  $\text{Ca}^{2+}$  levels (Biswas et al., 1999; Hu and Liu, 2011). Once mitochondria undergo challenges, a serial of responsive proteins will be differentially expressed to safeguard mitochondrial function (Valera-Alberni and Canto, 2018). In addition, the disturbance in mitochondrial dynamics due to long-lasting stress may trigger apoptosis in mitochondria. In this work, alterations of some stress-responsive and apoptosis proteins, including ATP-binding cassette (ABC), aldehyde dehydrogenases 2 (ALDH 2), sulfide quinone oxidoreductase (SQR), ATP-dependent RNA helicase DDX3X (DDX3X), stomatin-like protein 2 (SLP2) and voltage-dependent anion-selective channel 1 (VDAC1) were differentially expressed in Cd-treated clam samples. ABC belongs to transmembrane proteins and can drive the transport of various molecules across all cell membranes by binding and using ATP. It is usually associated with stress resistance (Carmona-Antonanzas et al., 2015; Chavan et al., 2011). The elevation of ABC implied that Cd treatment caused mitochondrial stress in clams. SLP2 functions as regulating the stability of specific mitochondrial proteins, such as respiratory chain complexes (Da Cruz et al., 2008). The up-regulation of SLP2 suggested the instability of ETC complexes induced by Cd, which were confirmed by the alterations of OXPHOS proteins mentioned above. It is noteworthy that up-regulations of ABC and SLP2 were detected in clams from both Cd treatments, indicating that these two proteins might be preferable biomarkers of stress-response to Cd exposure. Other DEPs, such as ALDH2, SQR and DDX3X, were only found to be altered in the low concentration (5  $\mu\text{g/L}$ ) of Cd-treated clam mitochondria. ALDH2 acts as an aldehyde scavenger during lipid peroxidation caused by stress and has ever been found to be elevated in men exposed to high levels of air pollutant like particulate matter (Singh et al., 2013; Winckelmans et al., 2017). The up-regulation of ALDH2 confirmed that there indeed existed stress in clams caused by Cd. SQR is a highly conserved enzyme involved in sulfide metabolism (Brito et al., 2009). It has been reported that SQR played a critical role in Cd detoxification in *Schizosaccharomyces pombe* (Kennedy et al., 2008). DDX3X is a highly conserved member of ATP-dependent DEAD-box RNA helicases and plays a key role in regulating the mitochondrial stress response (Padmanabhan et al., 2016). In our case, however, neither SQR nor DDX3X showed expected increases in Cd-treated clams, implying the possible dose-dependent effects of Cd to clams.

It is well accepted that apoptosis is closely associated with mitochondria. VDAC1 locates in the mitochondrial outer membrane forms hydrophilic pores and is the main gateway for the entry and exit of the mitochondrial metabolites (Colombini et al., 1996). Apart from being involved in energy metabolism, VDAC1 has an important function in apoptosis (Tomasello et al., 2013). The down-regulated VDAC1 in clams revealed the inhibition of apoptosis in 5  $\mu\text{g/L}$  Cd-treated clams. A previous study, however, gave an inconsistent result that higher concentrations (>3 mg/L) of Cd significantly induced apoptosis in clams *Meretrix meretrix* (Xia et al.,

2016).

The unexpected expressions of proteins including SQR, DDX3X, and VDAC1 implied the dose-dependent effects of Cd on stress resistance and apoptosis in clams. Treatments containing more concentrations of Cd are necessary to further characterize the mechanisms of mitochondrial stress resistance and apoptosis.

## 4. Conclusion

In this work, multiple biological processes in mitochondria, including TCA cycle, oxidative phosphorylation, fatty acid  $\beta$ -oxidation, stress resistance and apoptosis and mitochondrial fission, were significantly influenced by Cd exposure for 5 weeks. Furthermore, a significant decrease of mitochondrial membrane potential ( $\Delta\Psi_m$ ) and mitochondrial elongations were also observed in Cd-treated clam gills. These findings confirmed that mitochondrion was one of the key targets of Cd toxicity, and the dynamical regulations, such as reconstruction of energy homeostasis, induction of stress resistance and apoptosis, and morphological alterations, in mitochondria might play essential roles in Cd detoxification. Overall, this work presented a global view of mitochondrial responses of clams to Cd, contributing to better understanding the toxicological mechanisms of Cd.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2019.05.046>.

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