International Journal of Biology; Vol. 11, No. 3; 2019
ISSN 1916-9671 E-ISSN 1916-968X
Published by Canadian Center of Science and Education

# Toxic Effects of Domoic Acid on *Caenorhabditis elegans* and the Underlying Mechanism

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Received: March 25, 2019 Accepted: April 23, 2019 Online Published: May 4, 2019

doi:10.5539/ijb.v11n3p1 URL: https://doi.org/10.5539/ijb.v11n3p1

**Abstract**

Domoic acid (DA) is a well-known marine bio-toxin and has been investigated extensively through many in vitro and in vivo studies. However, the mechanism for DA-induced toxicity is still not fully understood. In the present study, we performed toxicity assessment of DA in *Caenorhabditis elegans* system and investigated the underlying mechanism for DA-induced toxicity. Our data show that DA exposure has toxic effects on locomotion behavior, lifespan and intestine of nematodes. Moreover, we observed the increased reactive oxygen species (ROS) formation in DA exposed nematodes, implying the involvement of oxidative stress in DA-induced toxicity. In addition, our data demonstrates that p38 signaling pathway is required for the DA-induced ROS formation and toxicity. Therefore, our study proves the effectiveness of *C. elegans* system in toxicity assessment of DA, and DA may induce toxicity through p38 pathway dependent oxidative stress.

**Keywords:** domoic acid, toxicity, *C. elegans*, p38 signaling pathway, oxidative stress

## 1. Introduction

Harmful algal blooms (HABs) and their potential toxic effects have become a focus of public concern (Grattan et al., 2016). Domoic acid (DA) is a well-known bio-toxin produced by marine algae such as diatom genus *Pseudo-nitzschia* (Hiolski et al., 2016). Many DA poisoning events have been noticed and recorded since 1987, when 4 people died and more than 100 people got ill after consuming DA-contaminated mussels (Govenkar & Wahidulla, 2000). High-dose DA can cause the clinical syndrome “Amnesic shellfish poisoning”, with symptoms including memory loss, seizures and even death (Grant et al., 2010). The structure of DA is highly similar to glutamate, allowing DA to bind to glutamate receptors including AMPA and NMDA receptors, thus leading to neurotoxic effects (Luana et al., 2018).

Several in vitro and in vivo toxicological studies against DA have been performed. For example, DA treatment led to neurotoxic syndrome and affected the locomotion activity in rats and mice (Grant et al., 2010; Jandová et al., 2014). Besides Central Neuvous System (CNS), which is the main target affected by DA exposure, heart has also been found to be affected by DA in rats (Andres et al., 2016). In addition, several studies reported that DA exposure induced neurotoxicity in fish (Salierno et al., 2006; Lefebvre et al., 2007). Giordano et al. reported that DA exposure induced cell death in mouse cerebellar granule neurons (Giordano et al., 2006). Despite the extensive studies on toxic effects of DA, the mechanism for DA-induced toxicity is stilled not fully understood.

Nematode *Caenorhabditis elegans* (*C. elegans*), a classical model organism used in biomedical research, has been widely accepted as a useful alternative animal model for toxicity evaluation (Leung et al., 2008). *C. elegans* is suitable for toxicological research because of its properties including short life cycle, large brood size and ease of culture (Qu et al., 2011). Moreover, the abundant resources of mutant nematode strains and well-established genetic techniques such as RNAi and transgenesis will help us explore the detailed toxic mechanisms (Yang et al., 2015). *C. elegans* assay system has been successfully utilized in toxicity assessment and toxicological study of many toxicants through multiple endpoints such as lethality, reproduction, lifespan, locomotion behavior and development (Ju et al., 2013; Zhang et al., 2011; Zhou et al., 2016a, 2016c). However, the toxic effects of DA have never been examined in *C. elegans*.

In the present study, we performed toxicity assessment of DA in *C. elegans* assay system. Moreover, we studied the underlying mechanism for DA-induced toxicity. Our data here will be helpful for the better understanding of DA-induced toxicity and maybe useful for the development of potential therapeutics against domoic acid poisoning in future.

## 2. Method

### *2.1 Worm Strains and Chemicals*

Domoic acid (purity ≧ 90%) was purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). The exposure concentrations of DA was 0.01-100 mg/L. Nematodes used in this study were wild-type N2, *pmk-1*(*km25*), *sek-1*(*km4*), *nsy-1*(*ok593*). All nematodes were provided by the *Caenorhabditis* Genetics Center (CGC). Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C (Sulston & Brenner, 1974; Zhang et al., 2015; Tissenbaum & Guarente, 2001). Synchronization of worm cultures was achieved using a bleaching buffer (0.45 M NaOH, 2% NaClO) treatment of gravid hermaphrodites (Zhao et al., 2013). Age-synchronized populations of L1-larvae or young adult nematodes were obtained by the collection (Donkin and Dusenbery, 1993). The exposure of worms was performed from L1-larvae to adult (prolonged exposure) in K-medium buffer (50 mM NaCl, 32 mM KCl) at 20°C incubator in the presence of OP50.

### *2.2 Lethality and Brood Size*

For the lethality assay (Wu et al., 2012), synchronous L1 worms were exposed in DA (0-100 mg/L) and OP50, nematodes were judged to be dead if they did not respond to stimulus. Approximately twenty nematodes were examined per treatment. The reproductive capacity was assayed by the brood size of adult nematodes (Bian et al., 2018). With one worm plate, eggs and larvae at all stages were counted and recorded. Worms were transferred daily to new agar plates, until the completion of the egg laying period. The hatched offspring were allowed to grow to L1/L2 stage and counted manually. Eight nematodes were examined per treatment. Three replicates were performed.

### *2.3 Locomotion Behavior*

Body bend, head thrash and basic movement were selected to analyze locomotion behavior (Zhuang et al., 2014). A head thrash was defined as a change in the direction of bending at the middle of body. To assay head thrash, each of the examined nematode was transferred into a microtiter well containing 60 μL of K-medium on the top of the agar, and head thrashes were counted for 1-min after a 1-min recovery period. A body bend was counted as a change in the direction of the part of the *C. elegans* corresponding to the posterior bulb of the pharynx along the *y* axis. To assay body bend, nematodes were picked onto a second plate and the number of body bends was scored at 20 second intervals. To assay basic movement, nematodes were picked onto an new plate without OP50 and scored the number of forward, backward, and U-turn movements within 20 sec. Eight nematodes were examined per treatment. Three replicates were performed.

### *2.4 Pharyngeal Pumping*

Methods were performed as described previously (Maria et al., 2019). The pharyngeal pumping rate was assessed with an Olympus microscope by observing the number of pharyngeal contractions during the 20 second interval. Eight nematodes were examined per treatment. Assays were repeated independently three times.

### *2.5 Lifespan*

For assay of lifespan (Zhang et al., 2014), synchronized L1 worms were exposed in plate containing DA and OP50. The L4 worms were counted and transferred daily to fresh plates until nematodes were dead. Survival curves and statistical analysis were performed with Graphpad Prism 5. Thirty nematodes were examined per treatment. For lifespan, graphs are representative of at least three trials.

### *2.6 Intestinal Autofluorescence*

Animals were fixed in M9 buffer (3g $KH_2PO_4$, 6g $Na_2HPO_4$, 5g NaCl, 1mL 1M $MgSO_4$, $H_2O$ to 1L) with 25mM levamisole and mounted on slides with 2% agarose pads. Images were collected for fluorescence by an Olympus microscope (365nm excitation/420nm emission). Intestinal autofluorescence was measured by determining the average pixel intensity in the intestine of each nematode using Image J Software (NIH Image, Bethesda, MD, USA) (Pincus et al., 2016). Eight nematodes were examined per treatment. There replicates were performed.

### *2.7 Reactive Oxygen Species (ROS) Determination*

The exposed worms were transferred to M9 buffer containing 2M of CM-$H_2$DCFDA to incubate for 3-h at 20°C (Wang et al., 2007). After washing three times with M9 buffer, the worms were mounted on agar pads in 0.5M

$NaN_3$. Fluorescence intensity (488nm excitation/510nm emission) proportional to ROS levels was observed using an Olympus microscope. The ROS production was expressed as relative fluorescent units (RFU) by Image J software (Zhang et al., 2013). Eight nematodes were examined per treatment. Three replicates were performed.

*2.8 Data Analysis*

Statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). All data were expressed as means ± standard error of the mean (S.E.M.). Student's t-tests were used for comparison of two treatments. Differences among different doses ($p<0.05$) were analyzed using ANOVA followed by Turkey's post hoc multiple comparisons test.

## 3. Results

*3.1 Effects of DA on Lethality and Reproduction of Nematodes*

We first examined the effects of DA exposure on lethelity of nematodes as it is a widely used endpoint in toxicity assessment of potential toxicants. After prolonged exposure to 0.01-100 mg/L of DA, we did not observe any lethality of nematodes (Figure 1A). Reproduction of *C. elegans* is often affected by toxicants as nematode reproduction system is one of secondary targeted organs for toxicants. We next investigated the effects of DA on reproduction of *C. elegans* using brood size as the endpoint. As shown in Figure 1B, DA exposure did not significantly affect the brood size of *C. elegans*.

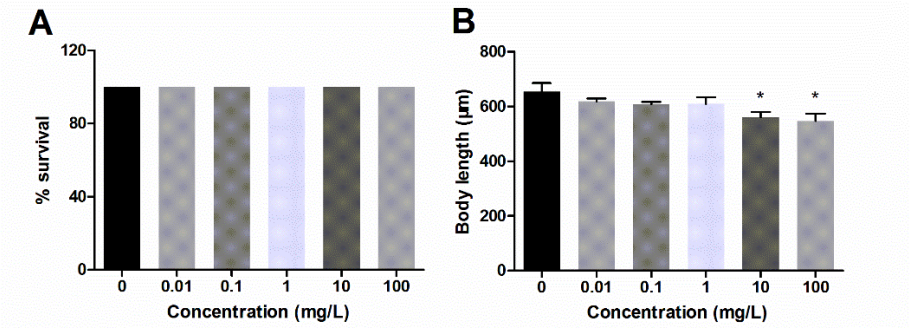


Figure 1. Effects of DA exposure on lethality and reproduction in wild-type nematodes. (A) Effects of DA exposure on the lethality. (B) Effects of DA exposure on the reproduction in nematodes. Exposures were performed from L1-larvae to adult (prolonged exposure). Bars represent mean ± S.E.M.

*3.2 Effects of DA on Locomotion Behavior of Nematodes*

Locomotion behavior of *C. elegans* is a useful endpoint in toxicity assessment of potential toxicants especially neurotoxicants as it is considered to reflect the effects on neurons. Thus, we examined the effects of DA exposure on locomotion behavior of nematodes. As shown in Figure 2A and 2B, prolonged exposure to DA at concentrations of 1-100 mg/L significantly decreased head thrash and body bend of nematodes. Moreover, exposure to DA at concentrations more than 10 mg/L significantly reduced forward movement of nematodes (Figure 2C). In addition, exposure to all the examined concentrations of DA significantly reduced pharyngeal pumping rates of nematodes (Figure 2D).

*3.3 Effects of DA on Lifespan and Intestinal Autofluorescence of Nematodes.*

We further examined the influence of DA exposure on lifespan of nematodes in order to investigate the long-term effects of DA on *C. elegans*. As shown in Figure 3A and 3B, exposure to DA at concentrations more than 1 mg/L significantly reduced the lifespan of *C. elegans*. We next examined the effect of DA exposure on nematode intestine, which is a major target organ for toxicants. As shown in Figure 3C and 3D, we found that exposure to DA at concentration of 10 mg/L significantly induced intestinal autofluorescence of nematodes, indicating that DA has toxic effects on intestine of *C. elegans*.

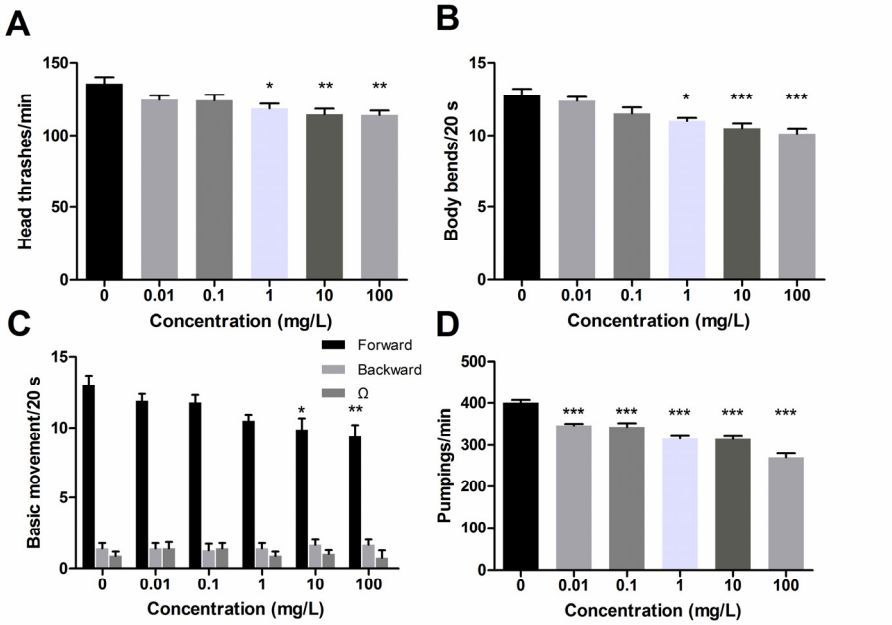


Figure 2. Effects of DA exposure on the locomotion behavior in wild-type nematodes. (A) Effects of DA exposure on the head thrashes. (B) Effects of DA exposure on the body bends. (C) Effects of DA exposure on the basic movement. (D) Effects of DA exposure on the pharyngeal pumping. Exposures were performed from L1-larvae to adult (prolonged exposure). Bars represent mean ± S.E.M. *$P<0.05$ versus control; **$P<0.01$ versus control; ***$P<0.001$ versus control

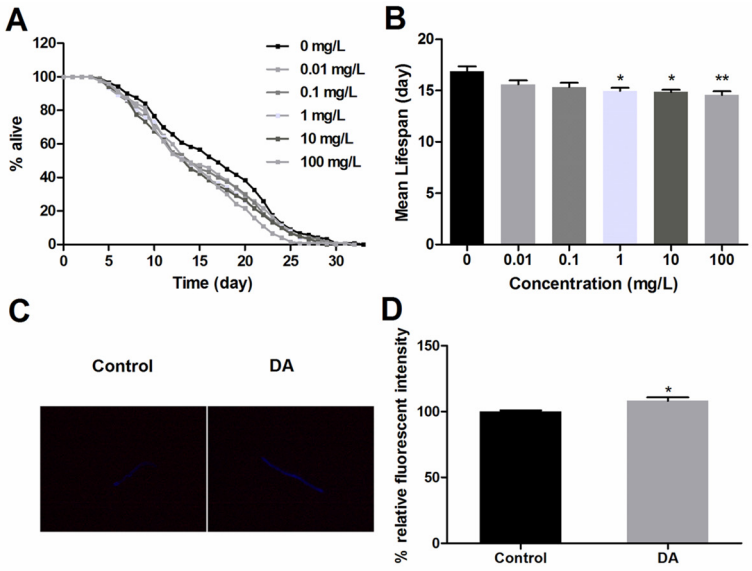


Figure 3. Effects of DA exposure on lifespan and intestinal autofluorescence of nematodes. (A, B) Effects of DA exposure on the survival in nematodes. (C, D) Effects of DA exposure on the intestinal autofluorescence. Exposures were performed from L1-larvae to adult (prolonged exposure). Bars represent mean ± S.E.M. *$P<0.05$ versus control; **$P<0.01$ versus control

### *3.4 Oxidative Stress is a Potential Mechanism of DA-induced Toxicity*

Oxidative stress is frequently induced by toxicants and is thought to be a common toxic mechanism in *C. elegans* (Chen et al., 2019). Thus, we investigated whether oxidative stress is involved in DA-induce toxicity in nematodes. We first examined the ROS formation in DA exposed nematodes. As shown in Figure 4A and 4B, 10mg/L of DA significantly induced ROS formation in *C. elegans*. Furthermore, treatment of antioxidant ascorbate acid

significantly ameliorated the locomotion behavior defects in DA exposed nematodes (Figure 4C, D). Taken together, oxidative stress may be a toxic mechanism of DA-induced toxicity.

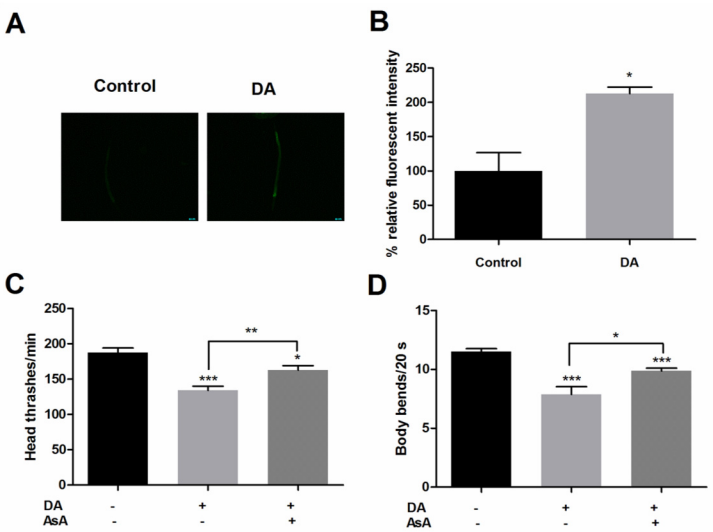


Figure 4. Effects of DA exposure on ROS formation in *C. elegans*. (A, B) Effects of DA exposure on ROS production. Prolonged exposure was performed from L1-larvae to young adults. (C, D) Effects of antioxidant treatment on locomotion behaviors of nematodes exposed to DA. 10mM of Ascorbate acid was added in DA-exposed L4 worms for 24-h. Bars represent mean ± S.E.M. *$P<0.05$ versus control; **$P<0.01$ versus control; ***$P<0.001$ versus control

*3.5 P38 MAPK Signaling is Involved In Regulating the Toxicity Induced by DA Exposure*

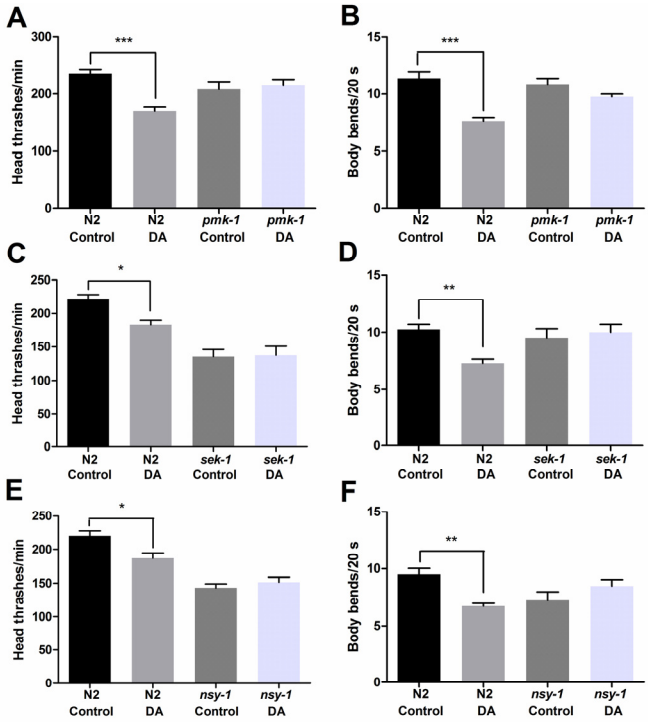


Figure 5. Locomotion behaviors in wild-type and mutants exposed to DA. (A, B) Effects of DA on locomotion behaviors in N2 and *pmk-1*mutant. (C, D) Effects of DA on locomotion behaviors in N2 and *sek-1* mutant. (E, F) Effects of DA on locomotion behaviors in N2 and *nsy-1* mutant. Prolonged exposure was performed from L1-larvae to young adults. DA exposure concentration was 10 mg/L. Bars represent means ± S.E.M. *$P<0.05$ versus control; **$P<0.01$ versus control; ***$P<0.001$ versus control

P38 MAPK signaling plays important roles in regulating diverse cellular processes including innate immunity, autophagy and cell growth (Cuadrado & Nebreda, 2018). P38 MAPK signaling has been found to be required for

ROS generation and plays an essential role in oxidatve stress regulation (Park et al., 2014). In *C. elegans*, PMK-1 (nematode ortholog of p38 MAPK) is found to be involved in toxicity induced by toxicants (Lim et al., 2012). To elucidate the function of PMK-1 in DA-induced toxicity and oxidative stress, we examined the locomotion behavior and ROS formation in mutant nematodes defective in PMK-1 MAPK signaling pathway. As shown in Figure 5A-5F, we found that mutant nematodes defective in PMK-1 p38 signaling pathway (*pmk-1*, *sek-1*, *nsy-1*) were resistant to DA-induced toxicity using locomotion behavior as endpoint. Furthermore, ROS formation induced by DA exposure was suppressed in pmk-1 and sek-1 mutant nematodes (Figure 6A-6B). There results indicated that p38 MAPK signaling is involved in regulating the toxicity induced by DA exposure.

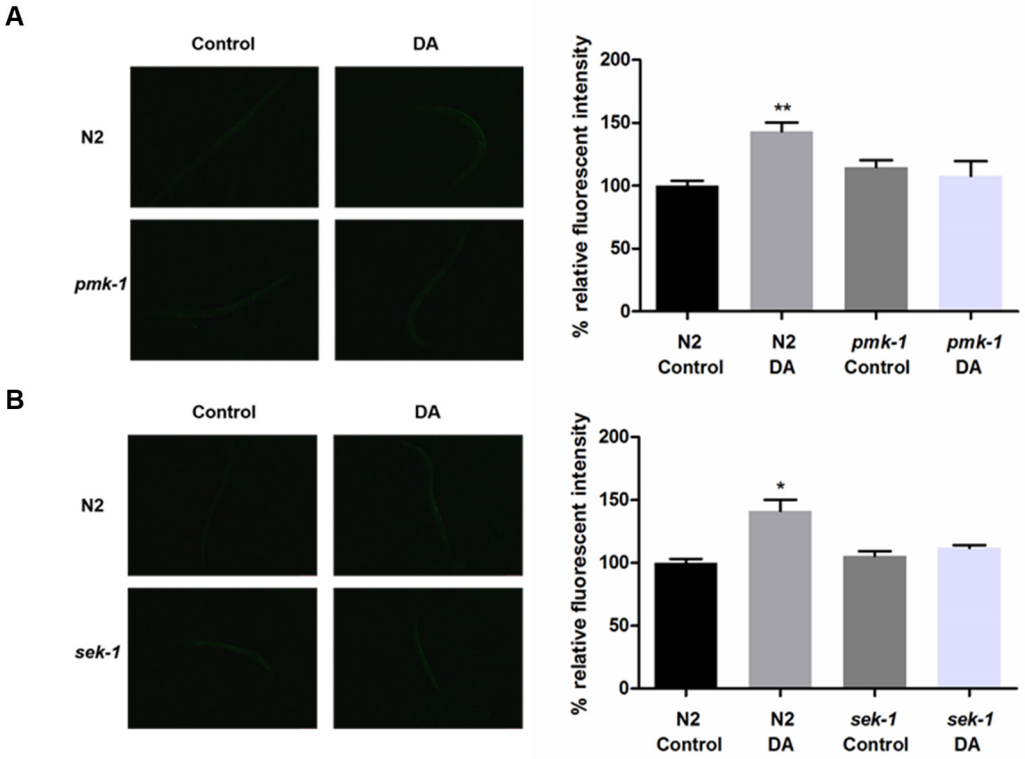


Figure 6. ROS production in wild-type and mutants exposed to DA. (A) Effects of DA on ROS production in N2 and *pmk-1*mutant. (B) Effects of DA on ROS production in N2 and *sek-1* mutant. Prolonged exposure was performed from L1-larvae to young adults. DA exposure concentration was 10 mg/L. Bars represent means ± S.E.M. *$P<0.05$ versus control; **$P<0.01$ versus control; ***$P<0.001$ versus control

## 4. Discussion

As a well-known marine toxin, the toxicity of DA has been studied extensively since 1987 (Perl et al., 1990). However, the mechanism for its toxicity is still not fully understood. In the present study, we demonstrated that *C. elegans* assay system is useful in toxicity assessment of DA. Although DA did not influence lethality and reproduction of *C. elegans* at the examined concentrations (Figure 1A-1B), DA exposure could induce the toxicity on locomotion behavior and lifespan of nematodes (Figure 2 and Figure 3A-3B). Moreover, our data showed that DA has toxic effects on nematode intestine (Figure 3C-3D), the primary targeted organ for toxicants of *C. elegans*, with the aid of intestinal autofluorescence as endpoint. Among all the endpoints used in this study, locomotion behavior of nematodes was found to be a sensitive endpoint for DA-induced toxicity. In *C. elegans*, locomotion behavior is considered to be a sensitive endpoint for neurotoxicity, which is consistent with the fact that DA is a neurotoxin. Thus, we chose locomotion behavior as the toxicity endpoint in the subsequent study.

In our effort to elucidate the potential mechanism for DA-induced toxicity, we observed increased ROS formation in *C. elegans* (Figure 4A-4B). Moreover, the toxic effects of DA on locomotion behavior of nematodes were suppressed by antioxidant treatment (Figure 4C-4D). These results imply that oxidative maybe a toxic mechanism for DA-induced toxicity. More importantly, our data demonstrated the effectiveness of antioxidant in the treatment of DA-induced toxicity.

Previous studies have found that p38 MAPK signaling pathway can be activated by DA and is involved in DA-induced apoptosis (Giordano et al., 2008). Thus, we wondered whether p38 pathway is involved in DA-induced toxicity. As shown in Figure 5A-5F, our data showed that *pmk-1*, *sek-1* and *nsy-1* mutant animals were resistant to DA-induced toxicity, implying that p38 signaling pathway is involved in DA-induced toxicity. The relationship between P38 signaling pathway and ROS formation has been widely studied. P38 can be activated by oxidative stress and acts downstream of ROS (Giordano et al., 2008). Also, p38 can be the upstream activator for ROS production under certain circumstances (Park et al., 2012). Thus, we investigated the relationship between p38 signaling pathway and ROS production during DA exposure. As shown in Figure 6A-6B, our results showed that DA-induced ROS formation was suppressed in pmk-1 and sek-1 mutant nematodes, implying that p38 signaling pathway is required for DA-induced ROS formation.

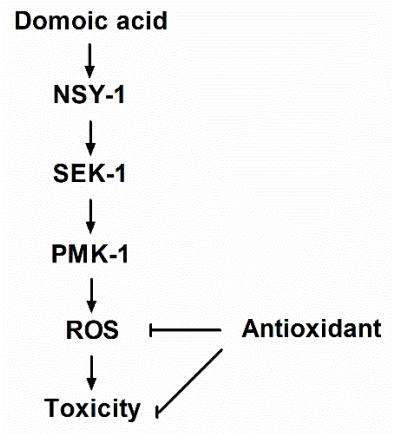


Figure 7. A model for the signaling pathway in the control of DA toxicity in nematodes

In conclusion, we prove the usefulness of *C. elegans* system in toxicity assessment of DA in the present study. We detect multiple toxic effects of DA exposure on nematodes *C. elegans* with the aid of locomotion behavior, lifespan and intestinal autofluorescence as the endpoints. Moreover, our data imply that oxidative stress is a toxic mechanism for DA-induced toxicity. Furthermore, we demonstrate that p38 signaling pathway is involved in DA-induced ROS production and toxicity (Figure 7). It is necessary to perform proteomic studies and find specific target proteins in signaling pathway by proteomics. Moreover, we can test the expression levels of proteins involved in signaling pathways to study the effects of DA. Our data will be helpful for the fully understanding of the mechanism for DA-induced toxicity and development of therapeutics against domoic acid poisoning in future.

**Acknowledgments**

We thank the *Caenorhabditis* Genetics Center for providing worm strains. This work was supported by National Natural Science Foundation of China (31300707), Changzhou Sci&Tech Program (Grant No. CJ20189001).

D.T. conceived and designed the experiments; D.T. and G.Z. performed the experiments; D.T. prepared the manuscript.

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