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Assessment of lead toxicity on locomotion and growth in a nematode *Caenorhabditis elegans*

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Abstract
Due to anthropogenic activities and natural abundance, lead exposure is a common phenomenon. Neurotoxic and genotoxic effects of lead are widely known. Recent studies have suggested that lead exposure can affect young generation and transfer to the progeny thus posing a great threat for future generation. The present study was focused on lead toxicity in terms of locomotion and growth of *Caenorhabditis elegans* (N2 wild type) at three sub-lethal doses (3µM, 15 µM and 30 µM) of Pb $(NO_3)_2$ for 24 hours (sub-chronic exposure). *Caenorhabditis elegans* is a nematode with an established eco- toxicity marker model organism, due to its short life cycle and ease to monitor. After lead exposure, significant toxic manifestations were observed in locomotion of the nematode in terms of omega bends (+350% for 30 µM exposure dose, $p<0.001$), reversals (-26.98%, -49% and -66.35% for 3 µM, 15 µM and 30 µM exposure doses respectively, $p<0.001$), turn counts (-38.66%, -62.61% and -81.93% for 3 µM, 15 µM and 30 µM exposure doses respectively, $p<0.001$) and peristaltic speed alterations (+97.83%, +225.92% and +454.63% for 3 µM, 15 µM and 30 µM exposure doses respectively, $p<0.001$). Successive reduction in the body length at lower doses shows remarkable toxic alterations in nematodes. The obtained data may be useful to extrapolate the effects of lead exposure on humans, as many of the similar pathways and cellular processes affected by Pb in humans are also present in *C. elegans*.

Keywords: *C. elegans*, Growth, Lead, Locomotion, Sub-lethal exposure

INTRODUCTION

Metallic elements with relatively high density develop toxicity at low concentrations, thus they are termed as pervasive and persistent pollutants in the environment. Lead, mercury and cadmium are high density metals, hence have the potential to cause adverse ecological effects. This is due to their characteristic of non-biodegradability, fast bio-accumulation than elimination that they are of high risk to living creatures found in the environment if present at higher concentration (Monnet-Tschudi *et al.,* 2006).

Lead (Pb), is a metal of global health concern especially in the developing countries. Known exposure routes include inhalation, ingestion, dermal absorption, retrograde axonal transport as well as transplacental route resulting in various adverse symptoms along with bio-accumulation (Rui and Wang, 2009). It can amass in the brain due to its ability to cross the blood- brain barrier (BBB) easily. It can preferably destroy the cerebellum, hippocampus and prefrontal cerebral cortex region of the brain (Tang *et al*., 2019). Almost all neurotransmitter systems (glutaminergic, dopaminergic, cholinergic) in the brain are reported to be affected by lead (Pohl *et al*., 2014). Blood levels of 0.48µmol/L can result in neurological disorders, cognitive impairments, hypertension and other disorders. Other deleterious effects of exposure includes impairment in kidney function, thyroid function deterioration, abnormal reproduction,

premature birth complexities and other neurodevelopmental defects in infants (Ruszkiewicz *et al*., 2018). Reports suggest that heavy metals such as lead present in industrial and household wastes have extensively polluted environmental components causing health hazards. It has also become a major public health concern worldwide hence their possible risk assessment for humans and the environment is essential to explore (Jiang *et al*., 2016).

Caenorhabditis elegans, an extensive, free-living nematode with a completely sequenced genome structure as well as having genetic similarity with vertebrates makes it a classic animal model system for biological studies (Choi, 2008). *C. elegans* has a short life cycle (3-4 days at 20°C), small size (1mm) and ease in breeding as well as monitoring behavior of the organism under microscope is feasible (Anderson *et al*., 2001). With stressful surroundings, nematodes can alter their growth and behavioral properties.

Established systematic assessment for toxicant exposure (with sublethal concentration) in *C. elegans,* it is mainly comprises of endpoints alterations like locomotion (Dayong and Xiaojuan, 2008), growth (Swain *et al*., 2004). Based on previous studies, various sensitive endpoints have been used to monitor effects of heavy metal exposure. Lead (Pb) toxicity has been examined on *C. elegans* after pretreatment with selenium, and locomotion (body bends, head thrashing and reversal frequency) was found to be mitigated (Li *et al*., 2013). Decreased locomotor activity based on body bending was also seen when L1 stage worms were exposed to 1.45mg/L of lead nitrate till adult stage (Ruszkiewicz *et al*., 2018).

Based on the previous studies, we have selected three sublethal doses of lead nitrate; 3µm, 15µm and 30µm respectively to investigate the respective toxic outcome of their exposure on locomotion (omega bends, reversals, turn count and peristaltic speed) and growth in the nematode model *C. elegans*.

MATERIALS AND METHODS

Experiment chemicals used: The $Pb(NO_3)_2$ was procured from Merck and sodium hypochlorite was obtained from SIGMA- ALDRICH. All other chemicals were purchased from SRL which were of analytical grade and high purity (99.9%). The distilled and Milli Q water was prepared using Lab India water purification system.

Strain maintenance and exposure: The experiment was done on wild type N2 strain of *C. elegans* obtained from Dr. Amir Nazir (CDRI-CSIR, Lucknow) as a gift. The strain was grown on Nematode Growth Medium and fed on *Escherichia coli* OP50 at 22°C (Brenner, 1974). The study was approved by University Research Committee (No. PhD/FS/RA/004). In present study Pb $(NO_3)_2$, the most abundant form of Pb exposure was used. For parameter analysis, age synchronized population was obtained using the modified method as described by Stiernagle, 2006 (bleaching the gravid animals with sodium hypochlorite bleach solution). The L4 stage worms (approximately 150-200 worms) were washed off using 1mL M9 and centrifuged (2000rpm,1minute) for locomotion analysis (Lewis and Fleming, 1995; Willhite and Mirkes, 2014) and 50-100 worms were used for each exposure. The L1 stage worms (approximately 150-200 worms) were similarly washed off, centrifuged for growth analysis and 50-100 worms were used for each exposure.

Parameter analysis: The goal of this study was to investigate the effects of Pb exposure on the *C. elegans* at lower sub-lethal doses and bring a consistency in dose related response of Pb toxicity at such doses. Instead of combination of metals, the present data provides baseline toxicity levels of lead exposure. Further, all the exposures were well under the LD50 dose of 421ppm for Pb toxicity in nematode (Williams and Dusenbery, 1988).

Locomotion: Age synchronized L4 stage worms were exposed to the three sub-lethal doses of Pb $(NO_3)_2$ (3µM, 15µM and 30µM prepared in Milli Q water) in a sterile 96 well plate in the absence of food and incubated for 24 hours at 22°C. Age synchronized L4 worms were placed in wells in the absence of food without any toxicant dose, incubated for the same time period and considered as control. Exposures of control and toxicants were carried out in triplicates. Compound microscope (micros AUSTRIA) attached with a tab was used for filming the videos. Locomotion was analyzed using WormLab (Version 3.0.0, MBF Bioscience, Williston, VT, USA)

Omega bends: Solution (20µL) from exposed well was taken out on a fresh non-seeded NGM plate and air dried. The worm was allowed to crawl away from any adherent food for one minute. The worm was then filmed under the microscope for three minutes where an omega turn was defined as when worm curls back with head touching the tail or crossing it during movement (Pierce-Shimomura *et al*., 1999). The procedure was repeated for nine animals chosen at random from each of the four groups.

Reversals: Few drops of 20µL worm solution from exposure wells was pipetted out and placed on a fresh non-seeded NGM plate and air dried. The worm was allowed to move away from any adherent food for one minute and then was filmed under the microscope for three minutes. Reversal was defined as anteriorly moving body wave forms during worm movement for several seconds and again moving forward in a new direction (Tsalik and Hobert, 2003). The procedure was repeated for three animals chosen at random from each control and concentrations.

Turn counts: Solution (20 µL) from exposed well was pipetted out and placed on a fresh non-seeded NGM plate and was air dried. The worm was allowed to move away from any adherent food for one minute. Then the worm was filmed under the microscope for three minutes. A body bend was defined as a change in the direction of a part of worm with respect to the posterior bulb of the pharynx along y-axis, presuming the worm was moving along x-axis (Pierce-Shimomura *et al*., 1999). The procedure was repeated for three animals chosen at random of each control and concentrations.

Peristaltic speed: Few drops of worm solution (20 µL) were taken out from the exposed well and placed on a fresh non-seeded NGM plate for air drying. The worm was allowed to move away from any adherent food for one minute and then filmed under the microscope for three minutes. Peristaltic speed was defined as the forward movement of the worm (peristaltic track length) with respect to the time moved by the worm (Chao, 2016). The procedure was repeated for three animals chosen at random of each control and concentrations.

Growth: Age synchronized worms were exposed to sub-lethal doses of toxicant $Pb(NO_3)_2$ (3 µM, 15 µM and 30 µM) with food (E. coli OP50) in a sterile 96 well plate and incubated for 48 hours at 22° C. Age synchronized L4 worms were placed in wells in the presence of food without any toxicant dose, incubated for the same time period and was considered as control. Each day 20µL of worm solution was taken out from one well and examined on an uncoated NGM plate. 20µL of sodium azide (25mM) was added over the drop to immobilize the worms and three worms chosen at random were photographed using compound microscope and analysed using Touch scope (Version 2.5.6). Treated and control nematodes were imaged using a microscope every day for two consecutive days.

Statistical analysis: The data from individual groups were presented as the mean ± standard deviation (n=9). Graphs were generated using Graph Pad Prism 8. One-way ANOVA test were performed between control and nematodes exposed to different doses of lead. A probability of less than or equal to 0.001 was considered to be significant and represents as ***.

RESULTS AND DISCUSSION

Because of high toxic effects, lead is a priority metal in the list of toxic metals and is of global health concern. Lead accumulates in tissues thus leads to multiple organ damage (Ruszkiewicz *et al*., 2018). Development of hypertension is reported in cases of occupational exposure of lead which may further contribute to impotence and other complications related to child birth (Wani *et al*., 2015). Lead is a neurotoxic pollutant that causes a number of degenerative Central Nervous System (CNS) problems which results in locomotion behaviour alterations in *experimental animals* (Rui and Wang, 2009, Adekomi *et al*., 2017).

Locomotion: Locomotion is an important endpoint in analysing the toxic effects of heavy metals in the nematode *C. elegans* (Anderson *et al*., 2001). Exposure of lead at concentrations 50 µM, 100 µM and 200 µM suppresses the body bends noticeably in *C. elegans* (Ye, Rui, Wu, and Wang, 2010). Dose dependent toxicity with alteration in locomotion behaviour of *C. elegans* has been observed in acute toxicity of lead (Dayong and Xiaojuan, 2008).

Omega bends: Omega bend is included as a locomotion parameter in the reversal frequency (Hart, 2006). It is an important indicator of neurological state of the nematode and its environment (Zhao *et al*., 2003). The results observed during the study revealed that there were no significant

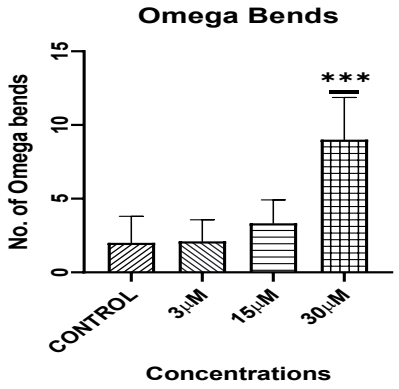

Fig.1. *Concentration- response graph for omega bend after exposure to Pb for 24 hours. Results are presented as mean ± SD. Asterisk (***) represented significant differences between control and exposed group (One-way ANOVA, p<0.001).*

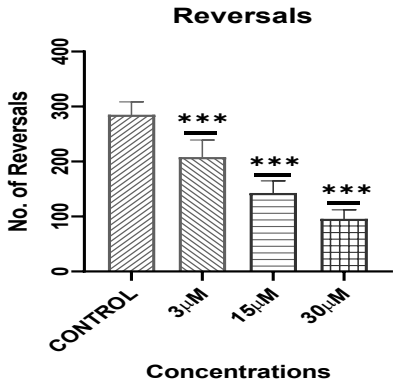

Fig. 2. *Concentration- response graph for reversals after exposure to Pb for 24 hours. Results are presented as mean ± SD. Asterisk (***) represented significant differences between control and exposed group (One-way ANOVA, p<0.001).*

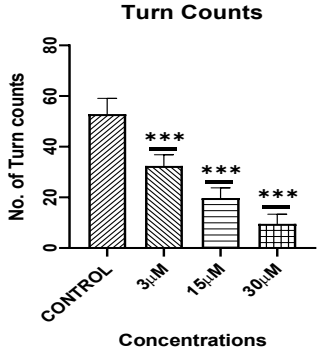

Fig. 3. *Concentration- response graph for turn counts after exposure to Pb for 24 hours. Results are presented as mean ± SD. Asterisk (***) represented significant differences between control and exposed group (One-way ANOVA, p<0.001).*

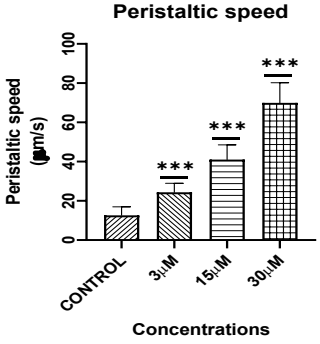

Fig. 4. *Concentration- response graph for peristaltic speed after exposure to Pb for 24 hours. Results are presented as mean ± SD. Asterisk (***) represented significant differences between control and exposed group (One-way ANOVA, p<0.001).*

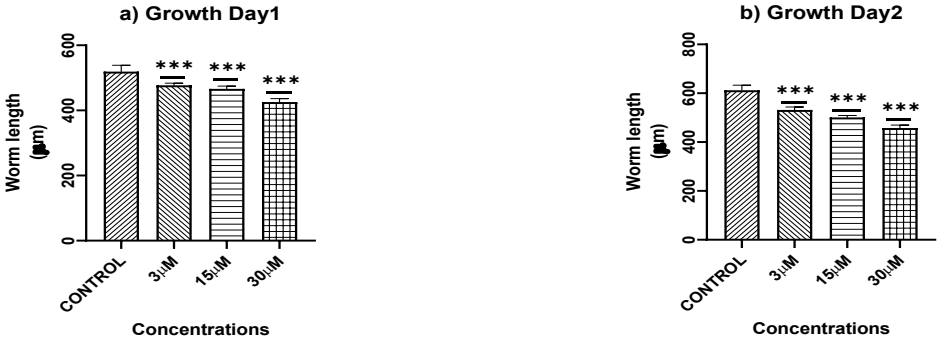

Fig. 5. *Concentration- response graph for growth after exposure to Pb for 24 hours a) on day 1 and b) on day 2 respectively. Results are presented as mean ± SD. Asterisk (***) represented significant differences between control and exposed group (One-way ANOVA, p<0.001).*

changes in the omega bends at 3 µM and 15 µM of $Pb(NO_3)_2$ however at 30 µM, there was a significant increase in the number of omega bends (350%, p<0.001) as compared to the controlled exposure (fig.1). Omega turn as an endpoint has been previously discussed in case of lead toxicity at variable doses in *C. elegans* (Dayong and Xiaojuan, 2008). But the present study has shown significant alterations which were not demonstrated in the earlier.

Reversals: The results of analysis reversals obtained are depicted in fig.2. Previously it has been reported that reversal frequency decreases in *C. elegans* when exposed to higher dose of 100 µM (Li *et al*., 2013). The worms exposed to $Pb(NO_3)_2$ showed a dose dependent decrease in reversal counts which was significant (p<0.001) i.e. 26.98%, 49% and 66.35% when exposed to Pb $(NO_3)_2$ at 3 µM, 15 µM and 30 µM respectively as compared to control.

Turn counts: Furthermore, the toxic effects of Pb $(NO_3)_2$ evaluated in terms of turn counts are given in Fig.3. Exposure to metals including Pb at 0 µM, 50 µM, 100 µM and 200 µM doses have been reported to suppress the body bend of the nematode *C. elegans* (Ye *et al*., 2010). Present study is in line with Ye *et al*., 2010 as our results shows significant decrease (p<0.001) of turn counts i.e. 38.66%, 62.61% and 81.93% when worms were exposed to 3 µM, 15 µM and 30 µM of $Pb(NO_3)_2$ respectively with respect to the controlled worms.

Peristaltic speed: Rate of movement can be an important endpoint in heavy metal toxicity analysis as suggested by Anderson *et al*. (2001). Fig.4 suggests significant increase (p<0.001) in the peristaltic speed of the exposed worms by 97.83%, 225.92% and 454.63% at to 3 µM, 15 µM and 30 µM of $Pb(NO_3)_2$ respectively as compared to the controlled worms. Results for the nematode *C. elegans* are not in line with the study carried out by Anderson *et al*., (2001) on *C. elegans*, as the rate of movement of nematode decreases with increase in toxic concentration of lead (study was conducted with food for 24 hours), while the exposure in present study was carried out without food in the wells. Further present results are in collaboration with (Angstman *et al*., 2016) obtained for *C. elegans* that the rate of movement of the nematode increases in the absence of food.

Growth: Lead exposed *C. elegans* are reported to show a range of abnormalities in their growth and development when exposed at 0.1µg/L and 10µg/L in successive generations (Yu *et al*., 2016). Larger larvae (L3) show better sensitivity in growth

parameter when exposed to lead (Yu *et al*., 2013). Fig.5a shows that on day 1, the body length of worms was decreased significantly ($p<0.001$) by 8.05%, 10.24% and 18.03% when exposed to 3 µM, 15 µM and 30 µM of $Pb(NO_3)_2$. Similar pattern was obtained on day 2. In comparison to the control, the decrease was 13.16%, 18.19% and 25.23% at $p<0.001$ with respect to the three doses of $Pb(NO_3)_2$ respectively as can be seen in fig.5b.

Intrusion of sub-chronic and low-level exposure of lead during early growth and development of human brain causes irreversible and non-curable damages. (Rogan *et al*., 2001). This susceptibility during the early childhood may finally lead to growth impairment in the individuals and experimental animal models. Comparison of growth data from day 1 verses day 2 revealed that due to the exposure of lead the growth of animal was arrested in dose dependent manner in terms of the rate of decrease in the body length of nematode. The growth in control group was significantly increased ($p<0.001$) i.e. 17.96% between day 1 and day 2. However, in 3 µM the growth was arrested by 36.47% in comparison to the control group. Further, both in 15 and 30 µM Pb exposed groups it was arrested by 58.24%, that indicates the tolerance or end point toxicity in terms of inhibition of growth of lead exposed nematodes.

Exposure of lead has been reported in escalated risk of premature child birth and abnormalities in their neurological developments (Anis *et al*., 2007). Ingestion of lead in rats can cause significant inflammation and irreversible CNS damage (Adekomi *et al*., 2017). Exposures with lead has been associated with conduct disorder and other criminal behaviour (Wang *et al*., 2008). Relationship between aggressive behaviour and lead toxicity has already been established (Li *et al*., 2003). Nevin, 2007 has reported reduced criminal activities over the time due to the omission of lead-based gasoline in United States. The present study shows behavioural and developmental alterations even at sub-lethal doses. It has been observed that these are early symptoms of neurological malfunction which may lead to severe abnormalities in later part of the life.

Although many endpoints have been studied in relation to the behavioural alterations in the nematode *C. elegans* but few locomotion parameters like peristaltic speed and omega bends have been hardly focused. Present study has brought out the importance of these locomotion parameters along with other previously known endpoints at such sub-lethal doses in sub-chronic exposure. Furthermore, endpoint toxicity with respect to growth in *C. elegans* has also been reported here to understand the health impairment due to lead exposure.

Conclusion

It is evident from the findings that reversals, turn counts, omega bends and peristaltic speed can be important endpoints for behavioural analysis of *C. elegans* in lead toxicity. Further tolerance or end point toxicity has been seen at a low sub-lethal dose of lead in the nematode *C. elegans.* The present data can also be used to extrapolate the effects of lead exposure on humans, as many of the pathways and cellular processes affected by Pb in humans are also present in *C. elegans*. As an overall basic outcome of the present study, it is of high concern that the use of lead needs to be regulated and reduced. Further alternatives are needed to be explored and put in common use to manage risk associated with lead toxicity.

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