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Lead acetate induced locomotor abnormality in Zebrafish embryo/larvae and amelioration by garlic aqueous extract

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Abstract
Lead is ubiquitous, non-biodegradable and highly toxic heavy metal. Lead exposure is estimated to account for 0.06% of the global burden of disease. Garlic, *Allium sativum L.* is a member of the Alliaceae family, is a popular remedy for various ailments. Hence in this study, the developmental toxic effects of lead acetate and its amelioration by Garlic aqueous extract (GAE) was evaluated by using a recently evolved test species, zebrafish. During the period 2017-18, normally dividing 5 hpf embryos were allotted into different groups, in six well plates. Two control groups one with plain embryo water and the other with garlic aqueous extract. Lead acetate was exposed at three dose levels 0.1, 0.5 and 1 ppm to next three groups. The other three groups were exposed with lead acetate at three levels along with 1 µg garlic aqueous extract. Behavioural changes were analyzed by kinovea software at 6 days post-fertilization. Lead induces significant dose dependent effect on the locomotor behaviour of the larvae. GAE has partial protective effect on lead acetate treated embryos. The amelioration by GAE was better in 0.1 ppm lead acetate treated group followed by 0.5 and 1.0 ppm lead acetate treated groups in the above mentioned parameters.

Keywords: Developmental toxicity, locomotor behaviour, garlic, lead acetate, Zebrafish

Introduction
Heavy metal pollution is posing a serious problem in India, affecting the environment and threatening the animal and human health [1]. Among them, lead is a well known non-biodegradable toxic heavy metal and now, it has become a global issue [2]. Lead also accumulates in food producing animals via contaminated feed, water and feed additives and may enter the human body through food chain and endanger human health [3]. Recently the use of lead in petrol, paint, plumbing and soldering have reduced which resulted in considerable reduction in lead levels in the blood [4]. But still in developing countries, there are notable sources of exposure to lead exist [4]. Lead is a cumulative toxicant that affects multiple body systems, including the neurological, haematological, gastrointestinal, cardiovascular and renal systems [4].

As lead usage has been placed under strict regulation in recent years, the likelihood of acute high dose exposure is significantly decreased. Hence, the current research focuses on chronic low dose exposure to lead and its asymptomatic nature [5]. The exposure to low lead level during developmental stage may result in mental retardation, impaired cognitive function, behavioural problems, and developmental delays and also associated with neurodegenerative disorders in later part of life [3]. According to WHO, lead is one of the ten chemicals causing major public health issue [6].

Garlic (*Allium sativum*) is one of the most commonly used plant, both for medicinal and culinary purposes [7]. Garlic is recognized to have incredible therapeutic and pharmacological properties like, antimicrobial, antithrombotic, antihypertensive, antiatherosclerotic, antihyperglycemic, antioxidant and anticancer. Many studies have also reported the prophylactic efficacy of garlic extract in reducing the lead burden from various tissues e.g. hepatic, renal, blood and bone [8]. A lot of health-associated features of garlic have been attributed to its main effective element organosulphur substance 'allicin' (thio-2-propene-1-sulfinic acid S-allyl ester) [9]. The Zebrafish, a robust tropical fish, has recently attained a pre-eminent position in biomedical research [10]. Zebrafish is considered as an ideal model for

assessing the developmental toxicity of exposure to toxicants during early-life stage. Its embryo assays are also regarded to be pain-free *in vivo* tests and are gradually being accepted as a good replacement for other types of animal experiment [11]. The Zebrafish possesses a number of strengths as a test species in developmental neurotoxicity studies including an abundance of embryos developing *ex utero*, presenting ease in chemical dosing and microscopic assessment at all early developmental stages. Zebrafish is also listed as a recommended test species in the 'Fish early life stage Toxicity test' (OECD Test guideline TG210) and the 'Fish short term toxicity test on embryo and sac-fry stages' (OECD Test guideline TG212) for determination of lethal and sub lethal effects of chemicals [12].

Hence in the present study developmental toxicity of lead acetate and its amelioration by garlic aqueous extract was evaluated using Zebrafish as a model organism.

Materials and Methods

Zebrafish and tank

Wild type Zebrafish (*Danio rerio*) were procured from local fish breeders and maintained in aerated standard fish rearing glass tanks. The stocking density was 5 fish/litre of water. Water without chlorination and reverse osmosis water in the ratio of 4:1 at the temperature of 29 °C, at a pH of 7.6 – 8.4, hardness of the water between 50 – 100 mg/L and electrical conductivity of the water between 360-520 µs was used for rearing the adult zebrafish. The temperature, pH and electrical conductivity of the zebrafish rearing water samples were estimated automatically by Multiparameter tester (PCST) in the department of Livestock Production and Management, Veterinary College and Research Institute, Namakkal. The hardness of the water was tested by EDTA titration method [13].

Rearing and Breeding of zebrafish

Male and female zebrafishes were maintained in separate tanks under the light: dark period of 14:10 hours and were fed with standard food pellets twice a day. Zebrafishes were fed with protein rich freeze dried worms one week before breeding. The male and female fish were separated one week before breeding and fed with protein rich freeze dried worms twice a day. A self-designed breeding tank was used. The mice cage was used as a breeding tank, where a window mosquito net was placed 1/3rd below the surface of water. Two sets of female and male zebrafishes in the ratio of 2: 1 were placed above the mosquito net, so that the adult zebrafish will not have the access to eat the eggs. The male and female fishes were placed in the breeding tank overnight. The eggs were laid in the morning following the first flash of light [13].

Egg collection and embryo water

The day one live fresh eggs of zebrafish were small, transparent and round in shape. The dead eggs were milky white in colour.

The live eggs were aspirated using pasture pipette and transferred to petridishes containing embryo water. The embryo water was prepared by adding 0.06 gram of ocean salt in one litre of reverse osmosis water. The collected zebrafish were washed twice in the embryo water. Normally dividing and spherical embryos at 5 hour post fertilization (hpf) were selected and utilized for the study [14].

Preparation of Garlic Aqueous Extract (GAE)

100 gram of peeled garlic was ground by adding 100 ml of cooled deionised water. The ground material was filtered through the filter paper to get the clear fresh extract. From this extract required concentrations of garlic aqueous extract (GAE) were prepared [15].

Dose fixation of garlic and lead acetate

The normally dividing embryos at 5 hpf (hours post fertilization) were exposed to different concentrations of GAE as follows, 1000, 100, 10, 1, 0.1 and 0.01 mg (20 embryos per concentration). Since the mortality was recorded up to 0.01 mg dose of GAE, based on the literature 1 µg of GAE was fixed as the dose [16].

The lead level used in the experiment was fixed based on the average estimated lead level in the poultry products (Egg/meat) of Namakkal district, Tamilnadu in the previous part of this study. Different concentrations of lead solutions and garlic aqueous extract were prepared using the embryo water.

Experimental Design

Normally dividing 5 hpf embryos were allotted into different groups as follows, in six well plates. Group I - Control (embryo water), Group II - Drug control (GAE 1 µg), Group III - 0.1 ppm lead acetate, Group IV - 0.5 ppm lead acetate, Group V - 1.0 ppm lead acetate, Group VI - 0.1 ppm lead acetate + GAE 1 µg, Group VII - 0.5 ppm lead acetate + GAE 1 µg and Group VIII - 1.0 ppm lead acetate + GAE 1 µg. Twenty embryos were allotted for a group with three replicates.

Behavioural study in Zebrafish

At 6 dpf a 15 minutes video of the larvae from each group was taken. The video was analyzed in the kinovea software as per its user manual to assess the locomotor behaviour of the zebrafish larvae (http://www.kinovea.org) [17].

Statistical analysis

The data were analysed by one way ANOVA procedure using SPSS® 20.0 software package for windows. Post-hock analysis was done by Duncan's significance difference test. The data on number of grids passed by the larvae in behaviour study was analyzed by Kruskal Wallis test followed by Mann Whitney u test [18].

Results and Discussion

Locomotor behaviour

Distance travelled by the larvae

The mean distance travelled by the zebrafish larvae observed at 6 dpf in the groups (I to VIII) are given in Table 1 and shown in fig.1.

Among the control groups, the larvae of garlic control group significantly ($P \leq 0.01$) travelled less distance compared to normal control group. All the lead alone treated groups (III, IV and V) differ significantly ($P \leq 0.01$) among themselves in dose dependent manner and also significantly travelled less distance when compared to control groups. The lead acetate and GAE treated groups VI, VII and VIII significantly ($P \leq 0.01$) travelled more distance when compared to lead acetate alone treated groups, III, IV and V respectively.

Number of grids crossed by the larvae

The mean number of grids crossed by the zebrafish larvae observed at 6 dpf in the groups (I to VIII) are given in Table 1 and shown in fig. 1.

There exists no significant difference between the control group and garlic control group. Among lead acetate alone treated groups, group V significantly (P≤ 0.01) passed less number of grids when compared to group IV and III. When comparing the lead acetate alone and lead acetate and GAE treated groups, there exists no significant difference between groups III, IV and V and groups VI, VII, and VIII respectively. But numerically there exists improvement in the number of grids passed by larvae of lead and GAE treated group when compared to lead alone treated groups.

Similar effects of lead acetate on locomotion of zebrafish larvae was reported by several authors [19, 20]. Lead significantly down regulated both neurexin 2aa and neurexin 2ab at 24, 48 and 72 hpf, which indicated that lead especially target neurexin 2a. The synaptic adhesion protein neurexin 2a plays a key role in neuronal development and locomotor behaviour [20]. Hence in this study, the dose dependant effect of lead acetate on locomotor behaviour of larvae may be due to the above proved reason.

Table 1: Lead acetate induced locomotor behaviour changes (Mean ± SE) in zebrafish larvae and ameliorative effect by GAE

S. No.	Groups	Distance travelled by the larvae (cm)	Number of grids passed by the larvae
1.	Group I	$4420.46^{h} \pm 0.97$	$22.50^{a} \pm 0.29$
2.	Group II	$3093.37^{g} \pm 0.49$	$21.50^{a} \pm 0.29$
3.	Group III	$1036.07^{e} \pm 0.35$	$10.75^{bc} \pm 0.25$
4.	Group IV	$476.08^{b} \pm 0.25$	$1.75^{de} \pm 0.25$
5.	Group V	$198.99^{a} \pm 0.22$	$0.75^{f} \pm 0.25$
6.	Group VI	$1088.30^{f} \pm 0.23$	$11.75^{b} \pm 0.25$
7.	Group VII	$932.37^{d} \pm 0.39$	$2.75^{d} \pm 0.25$
8.	Group VIII	$585.70^{c} \pm 0.76$	$1.50^{ef} \pm 0.29$

n = 4

*Overall mean bearing different superscripts between rows differ significantly ($P \leq 0.01$)

Amelioration by GAE

Garlic can prevent oxidative stress in lead toxicity by chelating lead ions and scavenging free radicals [21, 22]. Garlic was already proved to have protective effects against lead in rat [23, 24]. Garlic also proved to reduce the neuronal damage induced by lead [25].

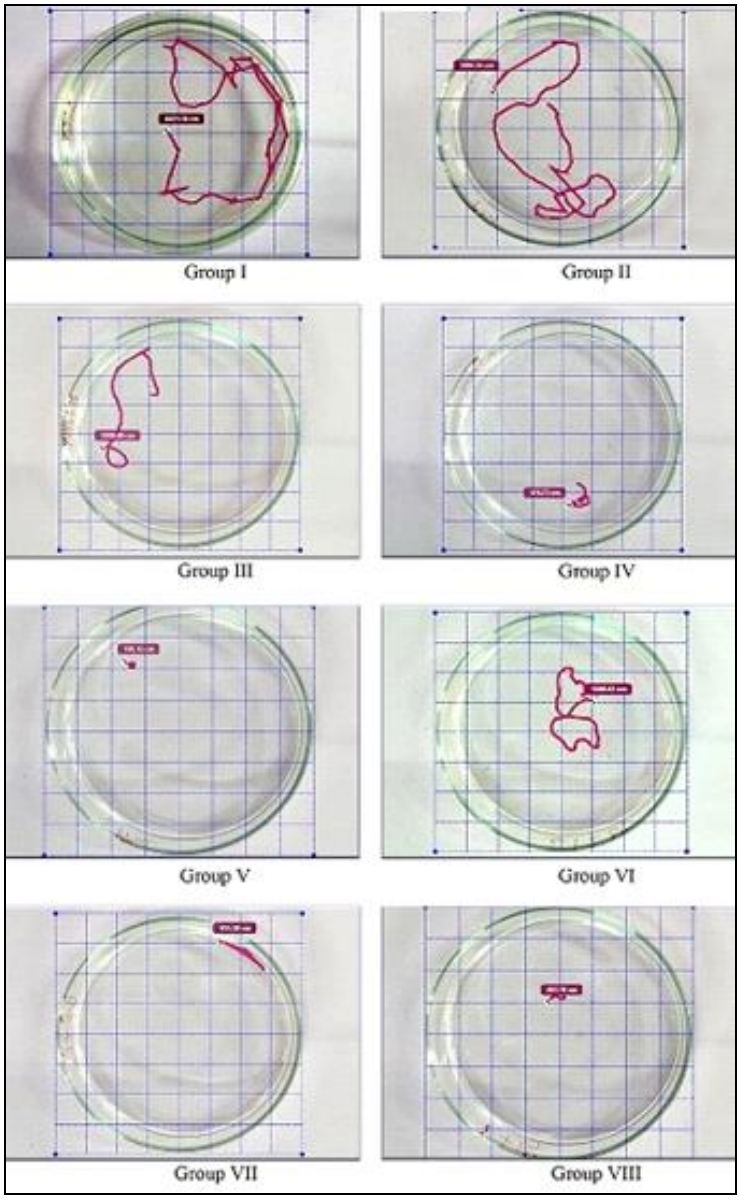

Fig 1: The figures showing the distance travelled and number of grids passed by the larvae of group I to VIII at 6 dpf

Conclusions

Lead induces dose dependant effect on locomotor behaviour in zebrafish embryo / larvae. GAE has partial protective effect on lead acetate treated embryos. The amelioration by GAE was better in 0.1 ppm lead acetate treated group followed by 0.5 and 1.0 ppm lead acetate treated groups respectively.

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