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Exploring the Role of Antioxidant Enzyme Activities in Reducing Oxidative Stress in the Earthworm *Pheretima Posthuma* Exposed to Heavy Metal Contaminated Soil

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Abstract: ***The present study of 96hrs was carried out to analyze the toxic effects of heavy metal lead nitrate (1.69 and 1.27 ppm) on anti-oxidative enzymes viz. Superoxide Dismutase, Catalase and Glutathione-S-transferase in Pheretimaposthuma. The worms for this observation were exposed to different concentrations of heavy meals viz. The lower and higher concentration calculated 1.69 and 1.27 ppm dry soil forlead nitrate for 5 days of exposure. A dose and time dependent increase in activities of these enzymes was observed. Lead nitratewas found to be more toxic at itshighest concentration.***

Keywords: Anti-oxidative enzymes, Super-oxide Dismutase, Catalase, Glutathione-S-transferase, *Pheretimaposthuma*

1. Introduction

Heavy metals constitute a group of elements which vary in their chemical as well as biological properties, as the name indicates these metals have specific gravity > 5 g-cm-3 (Holleman and Wiberg, 1985). Heavy metals kept under environment pollutant category due to their toxic effect on animals including human, plants and their food as well. Heavy metals have a property to persistent due to which they will get accumulate but not metabolized or breakdown into other intermediate compound. Hence these metals are accumulating in food chain through uptake at primary producer level and then through consumption at consumer level. The earthworms have the property to bioaccumulate the contamination such as heavy metals, pesticides *etc*. These worms are present at the base of food chain hence the understanding and the detail study of the effect of these pollutants (heavy metals) on earthworms is very essential for predicting the potential food chain impacts of soil contamination (Roubalova *et al.,* 2014). Earthworms have many interactions with the soil and due to these interactions the earthworms are significantly affected by the pollution originated by the rigorous use of heavy metals and biocides in agriculture, industrial activities, and atmospheric deposition. Hence earthworms have been proved as valuable bio-indicators of soil pollution (Lanno *et al.,* 2004).

In earthworm, defense system is present against lipid peroxidation (under stress condition) which is 'Antioxidant Defense System'. There are many important enzymatic scavengers of superoxide ion (O_2^{-}) and hydroxide ion (OH^{-}) such as SOD (Super Oxide Dismutase), CAT (Catalase), Glutathione-s-transferase. These enzymes act as scavengers by preventing the generation of hydroxyl free radical and thus protect the cellular constituents from oxidative damage (Scott *et al.,* 1991). The species, *Pheretimaposthuma* is an ideal species for predicting the effects of heavy metals on them due to the limited difference between their sensitivity to metals and the ease with which they can easily be reared and handled in the laboratory (Edwards and Coulson, 1992).

However, there is a lack of information on the effect of heavy metals on earthworms. In light of the above, the aim of this work was to characterize the antioxidant efficiency in terms of Superoxidative dismutase (SOD) Catalase and Glutathione-s-transferase of the earthworm, *Pheretimaposthuma* exposed separately to sub-lethal concentrations lead nitrate for 5days.

2. Materials and methods

The earthworm *Pheretimaposthuma* was selected as the test species for this experiment owing to its prevalence in Yashwant college Nanded, Maharashtra (India) in an area which has no history of input of either heavy metals or agrochemicals. *Pheretimaposthuma* is an anecic species, abundant and widely distributed in fertile land. Based on the results of 24 h toxicity tests (LC50), lower and higher sub lethal concentration of each heavy metal were chosen to study their impacts on enzymatic activity in different tissues content. The lower and higher concentration calculated 1.69 and 1.27 ppm dry soil for lead nitrate for 5 days of exposure. Soil was collected from an upland non-irrigated field, which had no record of input of agrochemicals (fertilizers and pesticides). The soil had the following characteristics: laterite type, sandy loam texture, pH-6.8, organic matter 2.7 g%, nitrogen 0.22 g% and a C/N ratio of 12.27. The soil was air dried and sieved before use. The earthworms were also collected from the above characterized field. They were cultured in their habitat soil and acclimated for one month with adequate provision of food (10% organic matter, cow dung leaf litter, moisture (20g %) and temperature (25±2ºC). Earthworms were removed from culture pots and kept half immersed in glass Petri plates containing 30ml of tap water in 25±2ºC temperature for 24 hours to evacuate their guts (Dash and Patra, 1977). The study was carried out in plastic culture pots under laboratory conditions following the

protocol of Panda and Sahu (2002). In brief, heavy metal copper sulphate used as the test chemicals was obtained from Ranbaxy Chemicals Ltd. The pesticides were chosen on the basis of their extensive use in this area.

The concentration were prepared in dilution of acetone and sprayed on the soil surface. After evaporation of the solvent, the treated soil was thoroughly mixed to distribute the pesticide evenly and enough water was added to bring the moisture content up to the field capacity. The same procedure with pure acetone was applied to prepare the controls. Ten healthy gut cleared earthworm were added to each pot. The experiment was maintained at 20 % soil moisture at 25±2ºC soil temperatures. Earthworm deaths were recorded and probit method of Finney (1971) was followed to calculate LD50 value for adult earthworm, Panda and Sahu (2002).

Superoxidative dismutase (sod):
SOD enzyme activity was assayed according to the method of Marklund and Marklund, (1974).

Procedure:
5% homogenate of tissues were prepared in Tris EDTA buffer and centrifuged for 40 minutes at 35000 at 4ºC assay mixtures contained in 1 ml volume.50 mm (pH 8.2 of TrisHcl buffer, 1mm diethylene Triaminepenta acetic acid (DTPA) 1mm Pyrogalol and 50 µl of enzyme. The reaction was started by the addition pyrogallol and the increase in absorbance at 420nm was noted over 30 sec. a blank was also run which had pyrogallol alone without the enzyme the changes in absorbance were noted one unit of SOD activity is the amount of the enzyme that inhibits the rate of auto oxidation pyrogallol by 50%. Activity of SOD was expressed as Unit/mg protein /min.

Estimation of catalase: (E. C.1.11.1.6)
Catalase activity was assayed by measuring the initial rate of disappearance of H_20_2 by the method of (Chance and Machly, 1955), 5% homogenate of tissue was prepared in cold phosphate buffer (pH 7.4) and centrifuged at 10000Xg for 15 minutes in refrigerated centrifuged. The supernatant was used for the enzyme assay. The 3 ml of reaction mixture contained 50µmoles phosphate buffer (pH 7.0) 51µlit. of 30% H_2O_2 and 100 µl of enzyme source for 50µl of tissue. The reaction tubes were shaken well and the absorbance was read at 260nm in UV spectrophotometer. The decrease in H_2O_2 was observed for about 2 minutes at 30 seconds interval. The activity of the enzyme was expressed in units, where one unit of catalase converts one mole of H_2O_2 /min.

Estimation of glutathione-s-transferas: (2.5.1.18)
Glutathione-S-Transferase (GST) was assayed using 1 chloro 2, 4dinitro benzene (CDNB) as the substrate according to the method of (Habig et al., 1974). GST is enzymes catalyzing reactions with glutathione as the first step in mercapturic acid synthesis. GST catalyses the formation of a thioether bond between reduced glutathione (GSH) and a large number of lipophilic compounds that possess an electrophilic center decrease in the absorbance of a substrate when conjugated with glutathione forms the basis of the spectophotometric assay. The reaction was started with the addition of enzyme. The reaction was continuously monitored at 37°C at 340 nm over a period of 5 minutes in U. V. spectrophotometer. The non-enzymatic reaction served as the blank. The amount of cytosolic protein added to the incubation mixture depended upon the tissue being analyzed the specific activity of GST was expressed as µmoles of thioether formed/mg protein/min.

Statistical analysis
All the reported results were expressed as mean of three replicates and all data so obtained was statistically evaluated using student "t" test.

3. Results

Super Oxide dismutase
SOD is an antioxidative enzyme which act first after the generation of Reactive Oxygen Species (ROS) and convert superoxide ions into peroxide (O_2 to H_2O_2). Dose and time dependent increase in SOD activity was observed in worms treated with concentration of lead nitrate (1.69 and 1.27 ppm).

Superoxide dismutase activities in the control worm *Pheretimaposthuma* of were found to be 0.310 units/mg proteins in skin; 0.289 units/mg protein in intestine; 0.319 units/mg protein in nephridia. Superoxide dismutase activities in lead nitrate (1.69 and 1.27 ppm) treated earthworm were found to be 0.290 and 0.285 units/mg protein in the skin; 0.278 and 0.275 units/mg protein in the intestine; 0.311 and 0.307 units/mg protein in the nephridia. Superoxide dismutase activities increased by 6.45% and 8% in the skin; 3.80% and 4.84% in the intestine; 2.50% and 3.76% in the nephridia. The increased rate of superoxide dismutase was statistically significant at ($P<0.05$).

Catalase
Catalase enzyme deactivates H_2O; hence prevent oxidative damage at cellular level. As H_2O_2 concentration increased due to increased activity of SOD on heavy metal exposure, hence dose and time dependent increase in CAT activity was observed in present investigation. Catalase activities in the control worm *Pheretimaposthuma* of were found to be 0.65 units/mg protein in skin; 0.40 units/mg protein in intestine; 0.72 units/mg protein in nephridia.

Catalase activities in lead nitrate (1.69 and 1.27 ppm) treated earthworm were found to be 0.40 and 0.28 units/mg protein in the skin; 0.25 and 0.22 units/mg protein in the intestine; 0.62 and 0.48 units/mg protein in the nephridia. The catalase activity, decreased by 38.46% and 56.92% in the skin; 37.5% and 45% in the intestine; 13.88% and 33.33% in the nephridia. The decreased rate of catalase was statistically significant at ($P<0.01$).

Glutathione-s-transferase:
Glutathione-s-transferase activities in the control worm *Pheretimaposthuma* of were found to be 0.38 µmoles of formazan liberated/mg protein/hour mg in skin; 0.30 µmoles of formazan liberated/mg protein/hour in intestine: 0.25 µmoles of formazan liberated/mg protein/hour in nephridia. glutathione-s-transferase activities in lead nitrate (1.69 and 1.27 ppm soil treated earthworm was found to be 0.21 and 0.18 µmoles of formazan liberated/mg protein/hour in the

skin; 0.19 and 0.14 µmoles of formazan liberated/mg protein/hour in the intestine; 0.13 and 0.11 µmoles of formazan liberated/mg protein/hour in the nephridia. Glutathione-s-transferase activities decreased by 44.73% and 52.63% in the skin; 36.33% and 53.33% in the intestine; 48% and 56% in the nephridia. The decreased rate of glutathione-s transferase was statistically significant at ($P<0.01$).

4. Discussion

Superoxide dismutase (SOD) enzyme constitutes on important link in the biological defense mechanism through disposition of endogenous cytotoxic superoxide dismutase (Fridovich, 1978). The present work indicates that SOD in the presence of H_2O_2 is involved in the generation of hydroxyl radical (OH), which property is responsible for its toxic effects. In the present study maximum decreases in SOD activity was recorded in skin of worms after exposures to in lead nitrate (1.69 and 1.27 ppm). Reduction in SOD activities coupled with elevation in lipid peroxidation suggests the exhaustion of this enzyme in a free radical rich environment due to heavy metal toxicity.

Catalase, which is found mainly in paroxysms, removes H_2O_2 produced during oxidation in that organelle. Catalase activity is known to alter under physiological and pathological conditions. In the present study maximum decreases in catalase activity was recorded in skin of worms after exposures to in lead nitrate (1.69 and 1.27 ppm).

In the present study, catalase activity decreased throughout the treatment span in all tissues in heavy metal treated. The reduction in the catalase activity could be due to an increased lipid peroxidation as results of toxicity. Sharma *et al*. (2020) reported Super oxide dismutase, Catalase and glutathioneperoxidase activity in heavy metals contaminated earthworm, *Eiseniafetida*. Ravi and Aruna (2010) reported antioxidant enzyme activities and markers of oxidative stress in the life cycle of earthworm, *eudriluseugeniae.* These Glutathione-S-transferases (GST) are particularly important in providing protection against xenophobic toxicity and oxidative stress. GST provide protection to the tissues by catalyzing the conjugation of wide variety of electrophilic xenobitics to GSH (Hebig*et al.,* 1974) and through noncatalytic binding of some of these compounds.

GST, isoenzyme remove a variety of organic hydro peroxides including free fatty acids hydro peroxides but not H_2O_2 presumably because H_2O_2 will not bind to the hydrophobic substrate binding site. According to Stadtman et al. (1992) glutathione and glutathione related enzymes protect against oxidative (free radical) cell injury. Stenersen et al. (1979) observed, glutathione related enzymes as one of the major detoxification and free radical scavenging systems may play a role in controlling the disease.

In the present study maximum decreases in nephridia of worms after exposures lower sub lethal concentration of in lead nitrate. Hans et al. (1993) reported GST has potential biomarkers term pesticide exposure. Similar trend of results was also observed by many scientists, according to Gaete *et al.* (2010) decrease in GST level was observed at highest concentration of mercury and the organisms which possess lowest oxidative damage was found due to increased protein content. At the same time activity of catalase also provide evidence against oxidative stress. The activity of GST increased in *E. fetida* when exposed to the higher concentrations of copper. Zhang *et al.* (2014) found that Imidacloprid could significantly stimulate the activity of CAT and SOD in earthworms. The Guadipyr could also enhance the activity of CAT, SOD and AChE in Daphnia *magna* Qi *et al., (*2013). Cao *et al*. (2017) reported oxidative stress induced by microcystins in *E. fetida* which led to lipid peroxidation and disruption of the antioxidant system. The increase in activity of SOD had a better protection against oxidative stress which might be due to increase in expression level of mRNA or the post transcriptional activation (Costa *et al.*, 1997). Increased concentration of heavy metal reduced the antioxidative enzymes activities in plants (Hou*et. al.*2007).

One of the main significance of this research would be to understand the mechanism of heavy metal toxicity and how it affects the physiology of soil organisms. Earthworms play a crucial role in soil ecology and are considered bioindicator species, meaning changes in their physiology can indicate changes in the overall health of the soil. Therefore, understanding the effects of heavy metal contamination on the physiology of earthworms can provide insights into the impact of heavy metal pollution on soil health.

Additionally, understanding how heavy metals affect the antioxidant enzyme activity and oxidative stress levels in earthworms may also be useful in developing strategies for mitigating the effects of heavy metal pollution on soil ecosystems. For example, if the study finds a specific antioxidant enzyme that is particularly affected by heavy metal exposure, then it may be possible to use that enzyme as a biomarker for heavy metal pollution in soil.

5. Conclusion

The conclusion of a study on the effects of heavy metal contamination on antioxidant enzyme activity and markers of oxidative stress in the earthworm *Pheretimaposthuma* would summarize the main findings of the research. It would discuss how the levels of heavy metals in the soil affected the antioxidant enzyme activity and markers of oxidative stress in the earthworms, and whether these effects were significant. Additionally, it would relate the results to other existing studies on the topic, and possibly to the ecological and environmental implications of the findings.

The conclusion would also suggest any further research that is needed. It could be like "The results of this study indicate that exposure to heavy metal contaminated soil leads to alterations in the antioxidant enzyme activities and increased oxidative stress in the earthworm *Pheretimaposthuma*. This highlights the potential ecological and environmental implications of heavy metal pollution on earthworm populations and the ecosystem services they provide. Further studies are needed to understand the mechanisms underlying these effects and to evaluate the long-term effects of heavy metal exposure on earthworm populations. "

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