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The Effects of Sub Lethal Levels of Lead on Acetylcholinesterase Activity in the Rock Pigeon, (*Columba Livia*)

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ABSTRACT

Lead is one of the most abundant heavy metals that has become widely distributed and mobilised in the environment. It exists in various forms which are bioavailable to many organisms including birds, causing physiological changes upon exposure. The aim of this study was to investigate the effects of sub lethal levels of lead on acetylcholinesterase activity in the Rock pigeon (*Columba livia*). Twelve free range and two pen reared (control) pigeons were collected from breeders around the city of Bulawayo and placed in separate cages. Six free range and one pen reared birds were exposed to 1.25 ppm lead acetate spiked feed and water for 72 hrs, whilst the remaining birds served as the unexposed control. Spiked feed and water were changed daily. Both the exposed and unexposed birds were sacrificed and the muscle, heart and liver tissues isolated and assayed for acetylcholinesterase activity. There was significant inhibition of enzyme activity in all tissue samples obtained from lead exposed free range birds when compared to their unexposed counterparts. In the lead exposed free range birds, muscle and liver acetylcholinesterase activity was below 0.005 µg/min/mg protein while that of the heart was in the 0.02-0.07 µg/min/mg protein range. Enzyme activity was greater than 0.1 µg/min/mg protein in the unexposed free range birds. Comparing the acetylcholinesterase activity of the lead exposed pen reared control, with that of the lead exposed free range birds, the latter demonstrated significant inhibition ($p<0.05$). The results therefore highlight the inhibitory effect of sub lethal levels of lead on acetylcholinesterase activity which could also be possibly enhanced by prior exposure to other environmental contaminants.

Key words: Acetylcholinesterase, inhibition, sub lethal, lead, exposure.

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1. INTRODUCTION

One of the most dangerous threats to the environment is pollution by heavy metals (Sevcikova et al., 2011). Heavy metals are defined as a group of metallic elements that have density higher than that of water (Tchounwou et al., 2012). Whilst they occur naturally in the earth, most environmental contamination results from anthropogenic activities such as mining, industrial production and agricultural use of metals and metal containing chemicals (Sevcikova et al., 2011, Tchounwou et al., 2012). Unlike organic pollutants, heavy metals are not biodegradable and so they tend to persist in the environment.

The ubiquity, persistence and accumulation of heavy metals in the environment implies that living organisms are continuously exposed to them, resulting in toxicity (Espín et al., 2014). Heavy metals such as lead may be present in soft acidic water or in air as particles, which are removed by rain or gravitational settling (Cao et al., 2003, Frantz et al., 2012). In the soil, lead is adsorbed strongly and retained in the upper soil layers (Setyorini et al., 2002). At this point, it presents a risk to wildlife, particularly birds such as pigeons, which deliberately ingest grit to help breakdown food (Begum and Sehrin, 2013, Frantz et al., 2012). The birds end up consuming lead, which accumulates in the blood, liver, kidney, bones as well as feathers, subsequently altering biological processes (Frantz et al., 2012). The biological changes that occur as a result, can be observed through the use of biomarkers, where a change in biological response related to exposure to environmental chemicals is measured (Peakall, 1994). One such biomarker is the esterase enzyme acetylcholinesterase (AChE).

Acetylcholinesterase belongs to the cholinesterases family, and is responsible for the breakdown of the neurotransmitter acetylcholine into choline and acetate (Lodish et al., 2000, Lionetto et al., 2013). Its activity terminates synaptic transmission, thus preventing continuous nerve firings at nerve endings (Lionetto et al., 2013). The inhibition of AChE has

been characterised as a biomarker of pesticide exposure, particularly the organophosphorus and carbamate compounds which specifically inhibit the enzymes' catalytic activity. In recent years, studies have shown sensitivity of AChE to other pollutants such as polycyclic aromatic hydrocarbons, detergents as well as heavy metals (Lionetto et al., 2013). Frasco and colleagues (2005) demonstrated the potential of metallic ions such as Hg^{2+}, Cd^{2+} and Cu^{2+} to depress or inhibit AChE activity. Acetylcholinesterase activity therefore serves as a good versatile biomarker.

While Rock pigeons accumulate lead in blood and tissues, they are resistant to its toxic effects, thus making the bird an excellent biomonitor of environmental lead (Dement et al., 1987). According to DeMent and colleagues (1987), all birds are constantly exposed to sub lethal quantities of lead. This study therefore was aimed at assessing the effects of sub lethal levels of lead on acetylcholinesterase activity in the Rock pigeon (*Columba livia*), a bird indigenous to Bulawayo, Zimbabwe.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals, substrates and enzymes were purchased from Sigma Aldrich Chemical Company, Germany. All other laboratory reagents were of analytical grade.

2.2 Maintenance of Rock pigeons and exposure

Twelve free range and two pen-reared (control) Rock pigeons used in this study, were obtained from breeders in the city of Bulawayo. These were caged separately and allowed to acclimatise to laboratory conditions for 7 days. Following acclimatisation, half of the free range or pen reared birds were exposed *ad libitum* to 1.25 ppm lead acetate for 72 hours, while the other half remained unexposed. The lead acetate spiked feed and water were changed every 24 hours. The lead acetate concentrations used for sub lethal exposure, were obtained from literature (Mehrotra et al., 2008).

2.3 Preparation of the post mitochondrial fraction (PMF)

Following the exposure period, the birds were sacrificed following the protocol outlined by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (AVMA, 2013). The birds were dissected and the liver, muscle tissue and heart were isolated. Each sample tissue was washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Individually, the samples were homogenized in five volumes ice cold 0.1 M potassium phosphate buffer, pH 7. The homogenates were centrifuged at 10 000 x g for 15 minutes at 4°C and the post-mitochondrial fraction collected and stored at -80°C prior to analysis.

2.4 Protein determination

Protein content of the post-mitochondrial fraction was determined following a modified method of Lowry et al., (1951) using bovine serum albumin (BSA) as a standard.

2.5 Assessment of acetylcholinesterase activity

Acetylcholinesterase activity was measured according to the method of (Ellman et al. (1961)) adapted for a microtitre plate reader as described by (Kallander et al., 1997). Briefly, the following reagents were added to the microtitre plate:110 µl of 0.01 M Tri/HCl buffer pH 8.0, 20 µl of 3.2 mM 5.5 dithio-bis-(2-nitrobenzoic acid) (DTNB) and 50 µl of 1 mg/ml PMF. The mixture was incubated for 3 minutes before adding 20 µl of 10 mM acetylthiocholine iodide. The rate of production of a complex between thiocholine and DTNB was followed at 25°C for 5 minutes at 412 nm using the SpectraMax 340 pc plate reader. Each sample was assayed in quadruplicate and specific activity calculated using an extinction coefficient of 14.15 $mM^{-1}cm^{-1}$.

2.6 Statistical analysis

Statistical differences were analysed using the one-way Analysis of Variance (ANOVA) Dunnet's Multiple Comparison test found in the GraphPad Prism 5 statistical analysis software program, purchased from GraphPad Software Inc. (La Jolla, CA, USA). The significance of the results was ascertained at $p<0.05$.

3. RESULTS

3.1 Effect of lead on muscle AChE activity

Acetylcholinesterase activity was significantly reduced ($p<0.05$) in the lead exposed free range birds, showing activity of less than 0.01 µg/min/mg protein as compared to the unexposed birds which had activity of above 0.025 µg/min/mg protein (Figure 1). In the pen-reared control, activity in the lead exposed birds was about 0.015 µg/min/mg protein, while that of the unexposed was approximately 0.03 µg/min/mg protein (Figure 1).

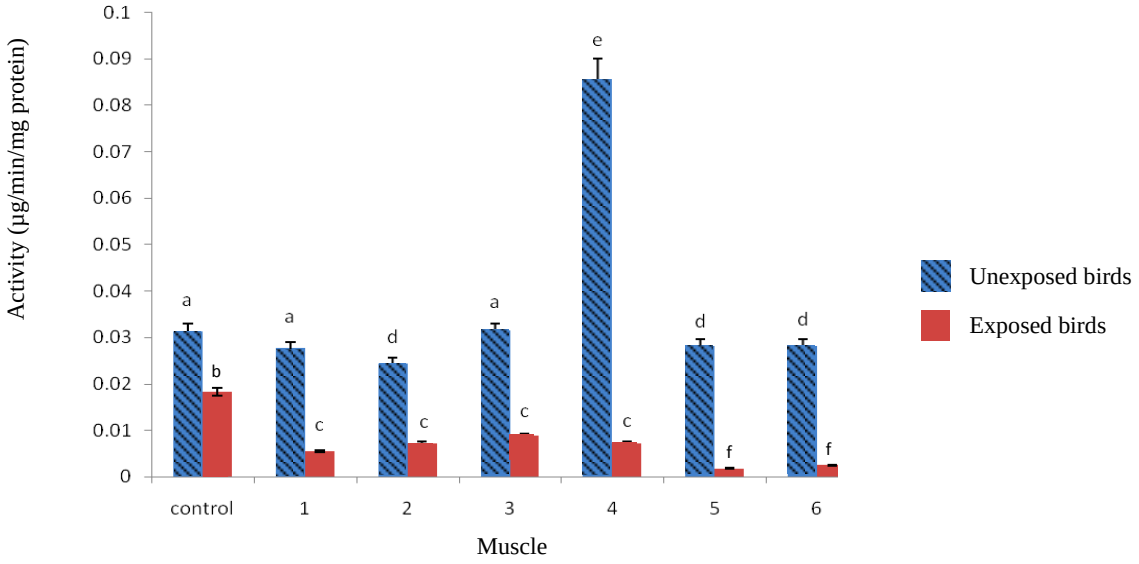

Figure 1. Effect of lead on acetylcholinesterase activity in muscle tissue of *Columba livia.* Control bars represent pen reared birds, while bars 1-6 represent free range birds. Values represent the average of quadruplicate measurement and are expressed as mean ±SD. Significance of the results was ascertained at *p*<0.05. Bars with different letters indicate significant differences and bars with the same letters indicate there was no significant difference.

3.2 Effect of lead on heart AChE activity

In both the free range and control pen reared birds exposed to lead, enzyme activity was low ranging between 0.02-0.07 µg/min/mg protein (Figure 2). On the other hand, acetylcholinesterase activity in all the unexposed birds was higher than in the exposed birds, in the 0.07-0.19 µg/min/mg protein range (Figure 2).

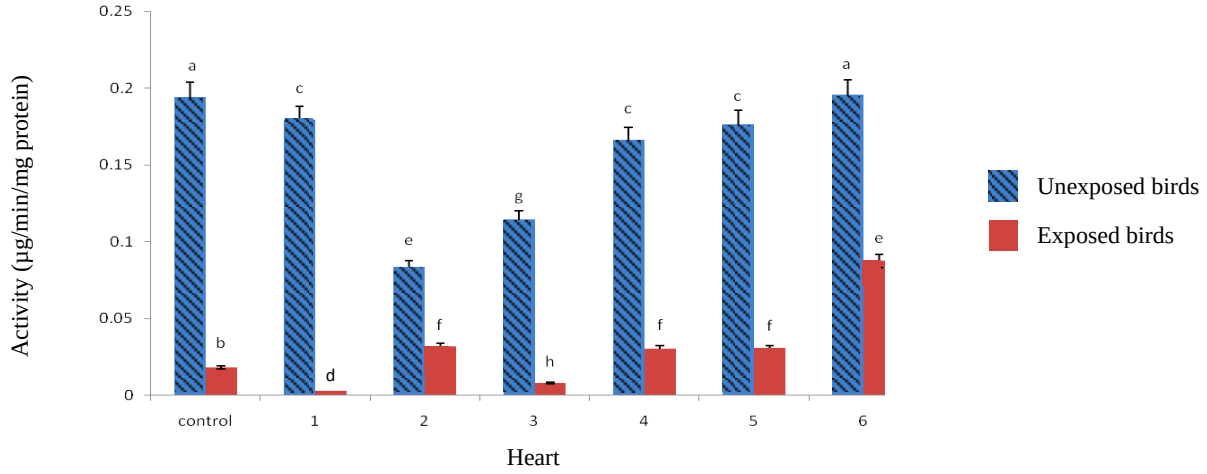

Figure 2. Effect of lead on acetylcholinesterase activity in heart tissue of *Columba livia*. Control bars represent pen reared birds, while bars 1-6 represent free range birds. Values represent the average of quadruplicate measurement and are expressed as mean ±SD. Significance of the results was ascertained at *p*<0.05. Bars with different letters indicate significant differences and bars with the same letters indicate there was no significant difference.

3.3 Effect of lead on liver AChE activity

Enzyme activity in the exposed free range birds was less than 0.02 µg/min/mg protein, while that of the unexposed birds was significantly high ($p<0.05$), ranging between 0.06-0.12 µg/min/mg protein (Figure 3). In the pen reared control, enzyme activity of the lead exposed and unexposed birds was 0.059 µg/min/mg protein and 0.062 µg/min/mg protein respectively (Figure 3).

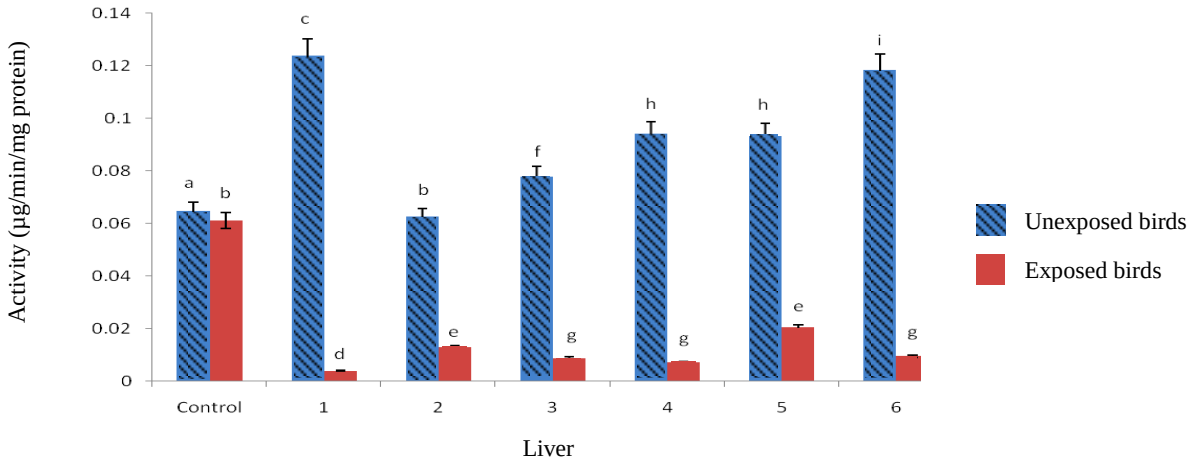

Figure 3. Effect of lead on acetylcholinesterase activity in liver tissue of *Columba livia*. Control bars represent pen reared birds, while bars 1-6 represent free range birds. Values represent the average of quadruplicate measurement and are expressed as mean ±SD. Significance of the results was ascertained at *p*<0.05. Bars with different letters indicate significant differences and bars with the same letters indicate there was no significant difference.

4. DISCUSSION

In the present study, acetylcholinesterase sensitivity in pigeons exposed to lead was observed. All sample tissues i.e. muscle, heart and liver from the exposed free range birds showed significantly reduced AChE activity as compared to the unexposed birds (Figures 1-3). These findings are in agreement with studies conducted by Hamidpoor and colleagues (2016) who observed significantly reduced AChE activity in the plasma of quail birds that had been exposed to lead. Reduced activity could be attributed to several reasons. It could be due to lead binding directly onto protein functional groups such as the sulfhydryl groups, thus compromising the enzymes catalytic activity (De Lima et al., 2013, Phyu and Tangpong, 2014). The differences in the magnitude of AChE inhibition in the different tissue samples (approximately 2.5, 5-10 and up to 18 fold reduction in activity for muscle, liver and heart tissue respectively) may have been due to AChE gene splice variants which give rise to multiple protein forms with differences in sensitivity (Lionetto et al., 2013, Resch, 2007).

The decreased enzyme activity may also be due to lead being a competitive inhibitor of the release of the acetylcholine neurotransmitter (Sadiq et al., 2012). Lead has the ability to mimic and inhibit the actions of calcium, which plays a role in the nervous system. Depolarisation of the presynaptic membrane causes calcium ions to enter the neuron, via voltage gated channels, and cause the synaptic vessels containing acetylcholine to fuse with the membrane and release the neurotransmitter (Lodish et al., 2000). Lead is absorbed through the same channels as calcium and so competitively inhibits the release of acetylcholine (Sadiq et al., 2012). With reduced amount of neurotransmitter released, there could result a corresponding reduced enzyme activity. To confirm this, however, electrophysical techniques that use intracellular recordings to monitor acetylcholine release would have to be performed. Using these techniques, Cooper and Manalis (1982) were able to demonstrate the competitive interaction between lead and calcium ions in their studies on the amphibian neuromuscular junction.

Apart from interfering with the release of acetylcholine, lead has also shown the ability to interact with acetylcholine receptors. Analysing rat brain tissue using ligand binding assays, Bondy and Agraul (1980) observed the inhibition of muscarinic acetylcholine receptors (which bind acetylcholine and mediate a metabolic response via second messenger cascades) by the interaction of its sulfhydryl groups with lead. Inhibition of the binding of acetylcholine would result in its accumulation, which may subsequently cause substrate inhibition of AChE enzyme (Reed et al., 2010). This may have caused the low activity observed in the current study.

Comparing the overall AChE activity of the exposed free range versus the exposed pen reared (control), the former showed significant decreased enzyme activity, more so in the liver and muscle tissues. This may be attributed to free range birds being exposed to a greater variety of pollutants as they fly around, as opposed to the pen reared that grow in controlled environments. These possible prior exposures may have caused a synergistic effect with lead, resulting in increased inhibition of AChE activity (Hamadipoor et al., 2016).

5. CONCLUSION

The present study shows that sub lethal levels of lead cause significant inhibition of AChE activity in muscle, heart and liver tissues of *Columba livia*. Further work may be carried out to assess AChE activity in other tissues e.g. gizzard, kidney etc. to get a wholesome picture of the enzymes' activity. Nonetheless, the inhibition of AChE here, demonstrated its worthy use as a biomarker of exposure to lead which could potentially be used in the monitoring of other heavy metals present in the environment.

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