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Detection of DNA Fragmentation in Liver of Goats Exposed to Lead Poisoning in Bagega District of Zamfara State, Nigeria

***Jubril A.T.[1], Fagbohun O.A.[2], Adekola A.A.[1]**

[1]Departments of Veterinary Pathology and [2]Veterinary Microbiology, University of Ibadan. Nigeria.

Summary: The ubiquitous presence of lead (Pb) and its release by anthropogenic activities has remained a major environmental pollution risk to both humans and animals. Lead toxicity has been associated with different systemic toxicities and biochemical impacts (such as oxidative stress and DNA damaging effects) with dire health consequences. In Nigeria, the health problem associated with lead toxicity has been overwhelming in the Bagega District of Zamfara State where artisanal gold mining has resulted in widespread environmental lead contamination. For this study, 24 goats were selected from two communities, 12 goats (exposed groups) selected from Bagega District, Zamfara (a community with widespread mining and lead contamination), while 12 goats (control) were selected from Apete, Ibadan with no previous mining history. The liver lead levels in the two groups were evaluated using the using Atomic Absorption Spectrophotometry and the lead level in the exposed group was categorized into 3 exposure categories (viz mild, moderate and severe). Representative liver samples from the 3 tissue lead exposure categories were analyzed using agarose gel electrophoresis for the detection of apoptotic oligonucleosomal DNA fragmentation. The tissue lead level in the goats from the exposed group (32.42±13.85 mg/kg) was significantly higher than the control group (0.36±0.12 mg/kg). DNA ladder was detected in the 3 exposure categories with a dose-related degree of DNA fragmentation. This study highlights the role of oligonucleosomal DNA fragmentation and apoptosis in the pathogenicity of lead in lead exposed goats and the associated the dose-gradient impact of tissue lead level on the degree of DNA fragmentation.

Keywords*:* Lead, Pollution, Liver, Apoptosis, DNA fragmentation

**Address for correspondence*: afusatjagun@yahoo.com; +2348034701005

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INTRODUCTION

Lead is a ubiquitous non-physiological important heavy metal pollutant in the environment. This heavy metal is widely distributed due to its relative abundance in nature and has found widespread use due to its desirable malleability and its relatively high resistance to corrosion (Landrigan et al., 1994; Needleman 2004). Important sources of heavy metals exposure include natural sources (e.g., water bodies, metal ores), industrial processes, commercial products, folk remedies, and contaminated food (Garza et al., 2006; Sharma et al., 2014).

Over the years, there has been a significant reduction in lead use however, the non-biodegradable nature of lead has accounted for its persistence in the environment and the continual release due to anthropogenic activities still makes lead pollution an environmental and public health concern (Elliot et al., 1999). The environmental contamination associated with lead is mostly due to its release by anthropogenic activities hence posing a significant risk to humans and animals due to their interaction and exposure to the same contaminated environment (Landrigan and Todd, 1994; Needleman, 2004; Akoto, et al., 2014). Livestock exposed to lead contamination also poses additional threat to public health due to the risk of heavy metal bioaccumulation with resultant toxic tissue lead residue above permissible consumption limit (Khalafalla et al., 2011).

In Nigeria, the Zamfara state lead toxicity outbreak is one of such cases of environmental lead contamination due to anthropogenic activities resulting from the indiscriminate artisanal small-scale gold mining and ore processing in the Bagega District of Zamfara. This incidence has led to the death of more than 400 children in the impoverished rural region and severely exposed thousand more to toxic lead dust levels (Plumlee et al., 2013). This has led to the reporting of this outbreak by both the Human Rights Watch (HRW) and Medecins Sans Frontiere (MSF) as the worst recorded case of acute lead poisoning in history (Burki, 2012; MSF, 2012). Therefore, there is a need for better understanding of the pathogenic mechanism involved in the toxicity as a means of stymieing future morbidity and mortality in the exposed population.

Lead due to its non-physiological importance to the body has been associated with a wide range of toxicities in the body. These effects are often seen in

the central nervous system, haemopoietic system, the portal of entry of the metal (gastrointestinal tract, respiratory tract), storage organ (liver and bone), excretory portals (kidneys) and the male and female reproductive organs (Valko et al., 2005). The clinico-pathological effects associated with these toxicities are intricately linked to the ability of the heavy metal to cause significant alteration in the normal body physiological and biochemical processes.

Lead and other heavy metals also utilize oxidative stress via the generation of reactive oxygen and nitrogen pro-oxidant species to induce hepatotoxic, neurotoxic, genotoxic and nephrotoxic effects in animals and humans (Patrick, 2006; Sharma et al., 2014). This oxidative stress mechanism is associated with the alteration of the antioxidant process via the inhibition of the activities of antioxidant molecules (such as GSH) and enzymes (such as superoxide dismutase, catalase, glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase). The combined effect of the generation of reactive oxygen species, reactive nitrogen species and alteration of the antioxidant mechanism thus aggravates the oxidative stress and the concomitant detrimental effects on the body physiological and biochemical mechanism (Sharma et al., 2014).

The lead toxicity-induced oxidative stress has also been associated with oxidative DNA damages such as strand break, generation of micronuclei, formation of DNA adduct (8-hydroxy-2-deoxyguanosine), and chromosomal aberrations (Ahmed et al., 2012; Koedrith and Seo, 2011). All these along with the direct impact of lead on the DNA repair enzyme also increase the potential for DNA damage accumulation. Hence, this results in genotoxicity, somatic mutation with potential oncogenic transformation and may the potentiate apoptosis in the affected cells (Koedrith and Seo, 2011).

Apoptosis or Type I programmed cell death (PCD 1) is a critical gene regulated cell death mechanism associated with both physiological (morphogenesis and physiological cell turnover) and pathological roles in the body. (Reed and Tomaselli, 2000; Elmore, 2007; Rastogi et al., 2009; Portt et al., 2011). Due to the sequential activation of caspases and substrate cleavage involved, apoptosis is associated with distinctive morphological and molecular (hallmark) changes (Wyllie et al., 2010; Portt et al., 2011; Favaloro, 2012). These changes include chromatin condensation, nuclear pyknosis, membrane blebbing, phosphatidylserine externalization, oligonucleosomal DNA fragmentation, and intracellular substrate cleavages. The expression of these hallmarks has thus been utilized extensively in the development of different tests and assays for the detection of apoptosis (Elmore, 2007; Wyllie et al., 2010; Portt et al., 2011).

The induction of DNA damage and the modification of some of the intrinsic biochemical and genetic mechanism because of lead toxicity have been proposed as an important trigger for the activation of the apoptotic mechanism. Therefore, apoptotic cell death has been suggested as an important mechanism for the expression of the toxicity of lead poisoning (Sharifi et al., 2002; Ahmed et al., 2013; Xu et al., 2015).

This study, therefore, evaluates the induction of apoptosis by lead toxicity using the detection of oligonucleosomal DNA fragmentation as a molecular biomarker and also examines the effect of dose variation on the extent of DNA fragmentation in lead exposed goats raised in Bagega District of Zamfara State.

MATERIALS AND METHODS

Study Area

Two districts in Nigeria were selected as sampling site for this study. This was done to ensure sampling from a region with environmental lead contamination issues (Bagega District, Zamfara State and this was tagged the lead-exposed district) and another area with no comparable prior history of contamination (Apete, Oyo State and this was tagged the Unexposed/Control site). The Bagega District of Zamfara (11.8648° N, 6.0024° E) is located in the Northwestern part of Nigeria. This district has been ravaged by lead environmental pollution and attendant acute lead poisoning outbreak due to the extensive illegal artisanal gold mining and ore processing being practised in the area. In contrast, Apete is a residential suburb in Ibadan, Oyo State (7.4493° N, 3.8721° E) with no prior history of mining activities and lead contamination predisposing industrial activities.

Animals

A total of twenty-four goats were selected from the goats slaughtered at the Community Slaughter slab from both districts. The goats sourced from Bagega district were classified as the lead-exposed group while those from Apete, Oyo State were classed as the unexposed group.
The animals selected for this study were adult of both sexes (average weight of about 10–15 kg) and were in apparent clinically healthy state. The animals were examined antemortem for obvious physical lesions and the assessment of their physiological parameters (heart and respiratory rates).

Sample Collection

Liver tissue samples were collected postmortem from the slaughtered animals and stored at -80°C until tested. This was done to retain the viability of the collected tissue sample for the evaluation of the tissue lead level and for DNA fragmentation assay using ethidium bromide-stained agarose gel electrophoresis

Evaluation of the liver lead level

The evaluation of the liver lead level was carried out (after wet digestion of the tissues) using atomic absorption spectrophotometry (AAS) as described by Julshman (1983) with slight modifications. Wet tissue digestion was carried out using the HNO_3 digestion method to obtain the wet tissue digest. The digested tissue samples were afterwards analyzed using an Alpha 4 Atomic Absorption Spectrophotometer, CHEMTECH 4200 for the tissue lead levels. The graphite furnace atomic absorption spectrophotometry readings and background correction (SR-BDG), signals were read at 283.3nm and all blanks, standards, and samples were analyzed in duplicates. The calibration curve gave a

linear response across this range with a correlation coefficient of t- test.

An arbitrary method decided upon by the researcher was used to stratify the category of lead exposure for grading and determining the possibility of a dose-response effect in the DNA fragmentation based on level of exposure. Hence, the liver lead level obtained above the FAO/WHO permissible tissue lead limit (0.5 mg/kg) (FAO/WHO, 2000) from the exposed were graded as mild (0.6 – 10.0 µg/g), moderate (10.1 – 20.0 µg/g) and severe (>20.1 µg/g) to obtain an exposure grading criterion for the goats.

DNA fragmentation assay using agarose gel electrophoresis

Representative goat liver tissue samples were selected from the three liver lead level groups (mild, moderate and severe groups) for agarose gel electrophoresis for the detection of DNA fragmentation. 50 mg of each liver tissue was homogenized in PBS to generate cell suspension. DNA was extracted from cell suspension using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA). 15 µl of the extracted DNA were then loaded onto a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in the gel ran at 85 V until the yellow dye in the loading dye ran to the edge of the gel. The ethidium bromide-stained DNA was then visualized by transillumination with UV light and photographed.

Statistical Analysis

The data collected were analyzed using SPSS v.24 and expressed as mean±SD. The student t-test and ANOVA were used for the comparison of the means and a p-value < 0.05 was accepted as statistically significant.

RESULTS

As shown in Figure 1, the tissue lead level in the unexposed group (0.36±0.12 mg/kg) was significantly lower than the exposed group level (32.42±13.85 mg/kg). The tissue lead levels in the exposed group animals (Table 1) were found to be very high and above the FAO/WHO permissible limits (0.5mg/kg). As such, the animals were categorized into 3 groups based on the severity of the exposure.

The lead level in the severe exposure group (58.65±14.45 mg/kg) was significantly higher than the values obtained in the mild (7.86±2.02 mg/kg) and mild exposure group (15.80±6.22 mg/kg). There was a lead-exposure level dependent difference in the degree of DNA fragmentation as shown in the agar gel electrophoresis (Figure 2).

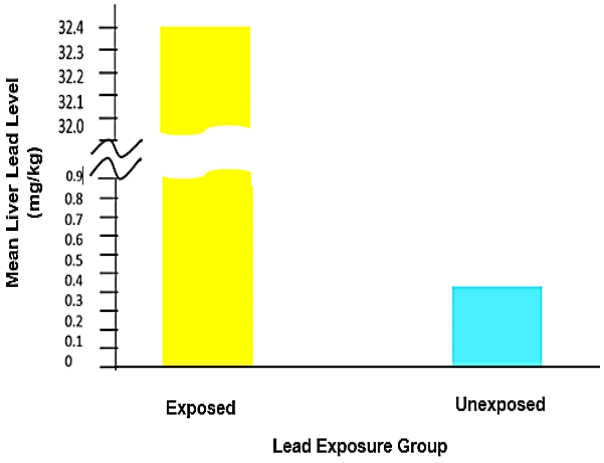

Figure 1: Bar chart of lead level in control and exposed

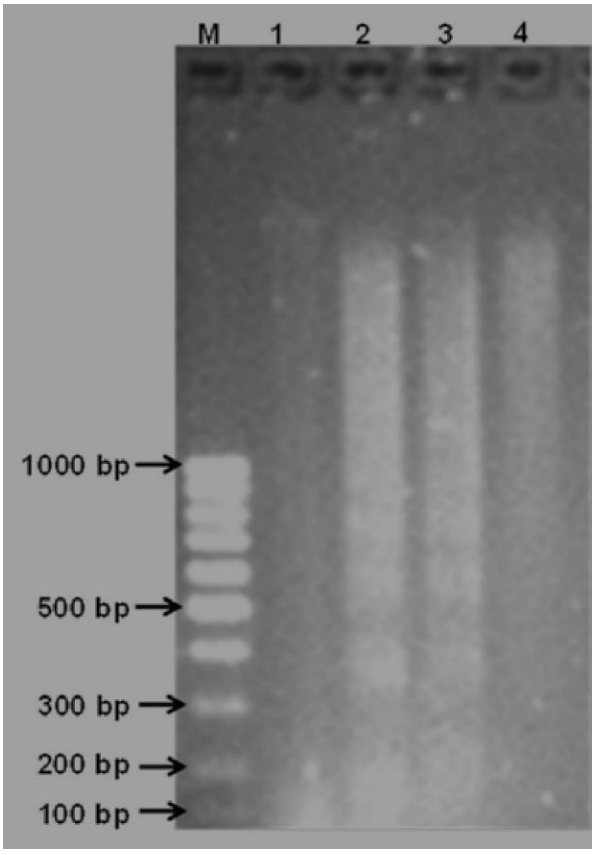

Figure 2: Agarose gel electrophoresis analysis of DNA fragmentation in liver. Effect of Pb on DNA fragmentation. DNA samples were extracted from liver tissues and DNA fragmentation was analyzed using 1.5% agarose gel electrophoresis. [Lane M, 1 kB ladder; lane 1, Liver 1(control); lane 2, Liver 2(severe exposure); lane 3, Liver 3(moderate exposure) and lane 4, liver 4(mild exposure)].

Table 1: Mean levels of lead in the liver of exposed goats

Category	Exposure Grading Criteria (mg/kg)	Number of animals (n)	Values (mg/kg)
Mild exposure	<0.6 – 10.0	3	7.86±2.02[a]
Moderate exposure	10.1 – 20.0	5	15.80±6.22[a]
Severe exposure	>20.1	4	58.65±14.45[b]

Means with the same superscript are not significantly different from each other (Tukey's HSD, P<0.05)

The highest degree of fragmentation was seen in the severe exposure category (lane 2) typified by a comparatively more distinct ladder shape compared to lane 3 and 4 which represent the goats in the moderate and mild exposure goat category respectively.

DISCUSSION

According to Swaileh *et al.,* (2004, 2009), different body organs have different propensity to concentrate different metals based on the calculation of the total rank score (TRS). The liver, kidney, lungs, small intestine heart and the brain in the order of arrangement based on this scoring system have been reported to be the major sites of heavy metal concentration. As a result, the liver due to its important role in metabolism and removal of toxic materials serves as a very important organ for the monitoring and assessment of the level of heavy metal exposure and associated pathology (Abou-Arab 2001; Akoto et al., 2014).

In this study, the lead level reported in the exposed goat was significantly higher compared to the unexposed goat group and this was consistent with findings from previous studies (Liu, 2003; Farmer and Farmer, 2000). This high liver lead level obtained in the exposed goats was similar to the findings reported by Liu (2003) in the soft tissues of sheep sampled from Baiyin in Gansu province of China (a non-ferrous metal smelting community where cases of strange lead toxicity-like diseases were reported in animals). Similar tissues lead levels were also reported by Farmer and Farmer (2000) in horses, cattle and sheep samples reared near a metal production area in eastern Kazakhstan. However, the lead levels reported in this present study was significantly higher than the level reported by Akoto et al. (2014) in free range sheep and goat reared in the Obuasi gold-mining town of Ghana.

Studies have shown that the genotoxic effects of lead could be ascribed to the effect of oxidative stress (arising due to the accumulation of reactive oxygen species and reactive nitrogen species and the alteration of the antioxidant system) on the DNA and the nucleic acids in the cell (Sharma et al.,2014; Koedrith and Seo, 2011). This thus results in significant changes to the cells genomic with changes such as DNA strand break, DNA protein crosslinking, chromosomal alteration, micronucleus and other genotoxic changes. Aside from the direct damage to the DNA and the genomic nucleic acid, there is evidence suggesting that oxidative stress mechanism is also involved in the reduction of the inherent body cell repair mechanism. The associated DNA damage seen in lead toxicity also serves as an important apoptotic trigger for apoptosis (Portt et al., 2011). More so, due to the ability to alter genomic DNA integrity, lead also possesses potential clastogenic effects along with the potential to exhibit oncogenic effects which could occur due to long-term lead exposure (Wise et al., 2004). The risk of lead-induced carcinogenesis has been associated with multifactorial causes which include oxidative stress (Jurczuk et al., 2006), altered cell signalling pathways (Wang et al., 2008, 2009), the induction of apoptosis (Xu et al., 2006, 2008) and the reduction in body cell repair mechanism.

As a consequence, DNA damage and apoptosis form a crucial component in the clinicopathology of lead poisoning and toxicity. Due to the unique molecular activities involved in the apoptotic process, the induction of irreparable DNA damage as an example of apoptotic trigger thus set up a cascade of substrate cleavage and caspase activation culminating in the morphological and biochemical hallmarks of apoptosis.

According to a study by Wolf et al. (1999), the manifestation of the different hallmarks of apoptosis, however, starts with the molecular activation different caspases and substrates. Oligonucleosomal DNA fragmentation is mediated by the activities of the Caspase-Activated DNase (CAD) (Enari et al., 1998; Liu et al., 1998). In healthy cells, this enzyme is normally complexed with the Inhibitor of Caspase-Activated DNase (ICAD). Activation of the apoptotic mechanism, therefore, leads to the activation of the apoptotic effector caspase, *caspase 3*, which results in the dissociation of the ICAD: CAD complex to release activated CAD. The oligonucleosomal fragments seen therefore is due to the cleavage of the DNA at the internucleosomal linker sites (which is the part exposed to the CAD) thus facilitating the oligonucleosomal fragmentation of the DNA of the affected cells at ~180-bp intervals (Enari et al., 1998; Liu et al., 1998). This characteristic DNA fragmentation into multiples of 180 – 200bps is responsible for the characteristic apoptotic DNA fragmentation (Wong, 2011) and this is similar to the presentation in lane 2 and 3 of this present study. This present study, therefore, points to the involvement of the Caspase-mediated apoptotic mechanism in lead toxicity in lead exposed goats.

In previous studies, the detection of the different hallmark of apoptosis, apoptotic regulatory proteins and cleavage products of apoptosis has thus been used experimentally as a measure of the severity of apoptosis and the inciting trigger (Yuste et al., 2001; Ahmed et al., 2013). Hence, this present study evaluated the dose-related impact of different tissue lead level on the degree of DNA fragmentation in lead exposed goats. This finding is similar to studies by Ahmed et al. (2013) conducted on the tissues of rats experimentally exposed to lead and assessed for varying levels of apoptotic markers such as caspase 8, caspase 9 and Bax in the liver, kidney and brain tissues.

This present study, therefore, accessed the effects of lead toxicity at the DNA level using agarose gel electrophoresis to monitor DNA fragmentation in a bid to

unravel the mechanism of lead-induced apoptosis in the hepatotoxicity and pathology of lead toxicity.

The detection of DNA ladder in this present study in the lead exposed goats demonstrate the relevance of apoptosis as an important mechanism in the pathologies and clinicopathological features of lead poisoning. This along with the dose-response variation in the level of DNA fragmentation seen with the different level of lead exposure also further strengthens the effect of the level of exposure on the severity of the apoptotic changes observed. This also makes DNA fragmentation detection an important adjunct diagnostic tool in the evaluation of the severity and extent of lead poisoning in toxicologic screening programmes and research.

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