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Haemotoxic and Genotoxic Potential of Lead on the Egyptian Toad *Amietophrynus regularis*

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Abstract: Many populations of amphibians are declining on all six continents on which they occur. The reason for the declines is a direct response to the habitat destruction and pollution including heavy metals. Heavy metals represent a major environmental problem of increasing concern. They are generally found at very low concentrations. They are difficult to remove from the environment and cannot be chemically or biologically degraded. Some heavy metals like lead seem to lack biological functions and extremely toxic even at low concentrations. This study was aimed to investigate the haemotoxic and genotoxic potential of lead using blood parameters, the frequencies of micronuclei, and nuclear lesions in erythrocytes of Egyptian Toad *Amietophrynus regularis* as biomarkers. The results of this work revealed that Pb was potentially accumulated in liver and muscles based on dose received. Toad exposed to the selected doses of lead produced dose – dependent significant increases in the concentration of lead in the liver and muscle, confirming the ability of *Amietophrynus regularis* to take up and accumulate heavy metals from their ambient habitat. The results of the present investigation showed that the lead treatment inflicted a drastic reduction in the means of RBCs, haemoglobin, and haematocrit values in addition to remarkable increase in WBCs, impairing the major blood parameters in this investigation. Correlation analysis has demonstrated a negative effect of Pb accumulation on RBCs count, haemoglobin, and haematocrit. Oppositely, Pb in muscles and liver exhibited a positive effect in WBCs count. In this study, higher incidences of micronuclei (MN) and nuclear lesions (NL) were found in the blood of toad exposed to lead doses. Such frequencies were significantly elevated with the increasing lead doses. A positive correlation was demonstrated between the investigated heavy metals in tissues and the induction of micronucleated RBCs and nuclear abnormalities in *Amietophrynus regularis*. The results of this study confirm the usefulness of the erythrocyte MN and NL as powerful monitoring tools for detecting genotoxic agents in aquatic and terrestrial environment.

Keywords: *Amietophrynus regularis*, Lead, Biomarkers, Heavy Metals, Genotoxicity, Micronuclei, Nuclear Lesions

1. Introduction

As a part of the ecological balance, amphibians are environmental sentinels for many habitats [1]. Amphibians are also important for ecological studies, giving information on the ecological impact of both local and global changes, sometimes with implication for humans [2]. Amphibians are important ecological components of both wetlands and dry land. Among vertebrates they are distinctive in many ways. For biological assessments, they are especially promising because of their capability of linking wetlands with surrounding landscape. A thin, moist, highly permeable skin; jellied, unshelled eggs; possession of aquatic and terrestrial life histories; restricted home range; and limited dispersal abilities of many species make amphibians as effective bio-monitors [3]. Dramatic changes in their populations and increased incidences of diseases and malformations, particularly in seemingly pristine areas, highlight concerns about general environmental deterioration. Over the last 50 years, many species of amphibians (frogs, toads, salamanders and newts) throughout the world have declined markedly in numbers [4, 5]. These declines raise the global eyebrow

because amphibians are indicators of ecosystem health [4, 6]. Some species have become extinct. The reason for the declines is a direct response to habitat destruction and chemical pollution including heavy metals [7].

Heavy metals represent a major environmental problem of increasing concern and their monitoring has received significant attention in the field and under laboratory conditions [8]. They are generally found at very low concentrations. The contamination of freshwater with heavy metals has become a matter of great concern over the last few decades because of the threat to public water supplies, their devastating effects on the ecological balance of the aquatic environment and their damage caused to the aquatic life [9]. Total heavy metal concentrations in aquatic components can mirror the present pollution status of these areas [10]. Because of their high degree of toxicity, some heavy metals are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure [11]. Lead Pb for example, can introduce into the environment via different ways; as dusts created by the weathering of deposits, steel and iron industries and from lead production and processing operations [12], sea and Salt Lake aerosols, forest fires, and volcanic eruptions [13]. Soil and bed sediments can hold large amounts of metals [14, 15, 16, 17]. High lead contamination in the range of 2000 mg/l in soil and 400 mg/l in river sediments in Egypt were reported [14, 15]. During hibernation toads can uptake metals form soils through their permeable surface [18].

Impact of pollutants, particularly heavy metals on the blood parameters of many aquatic fauna including amphibian have received a global attention. Recently, hematological variables have become promising biomarkers in measuring the effects of pollution, because blood parameters respond to low doses of pollutants. Blood parameters are important in diagnosing the structural and functional status of animal exposed to environmental pollutants. The micronucleus test (MN) developed by Schmid, [19] using mammalian bone marrow cells, has been applied extensively to test the genotoxicity of chemicals. Micronuclei are formed by the loss of whole chromosomes or portions of chromosomes from daughter nuclei at mitosis and exist separately from the main nucleus of the cell [20]. From the hematological point of view, heavy metals are known as one of the factors causing alteration in blood parameters [21]. Micronuclei as biomarker of pollution could be defined as genetic material fragments, due to the activity of clastogenic agents which provoke chromosome [22].

Nuclear lesions including binuclei are genotoxic analogues of micronuclei, which may also be the result of the action of a genotoxic agent [23]. These lesions were considered to be of genotoxic origin and used as a signal of cytogenetic damage in exposed animal. This study was aimed at the study of the haemotoxic and genotoxic potential of lead using blood parameters, the frequencies of micronuclei, and nuclear lesions in erythrocytes of Egyptian Toad *Amietophrynus regularis* as biomarkers.

2. Materials and Methods

2.1. Experiment Design

Amietophrynus regularis were procured from experimental animal house in Cairo. Upon arrival at the laboratory, toads were acclimatized to laboratory conditions for seven days. Toads of relatively similar sizes were selected (about 50g). [24]. There were no significant differences ($p>0.05$) between the mean weights 50±20g of toads used in the experiments. A Total of 30 toads (24 females and 6 males) were subjected to the experiment. Pb as lead acetate was used for the sub lethal tests. Toads were divided into six groups (5 individuals each): Three groups exposed to the three lead acetate concentrations (25, 50, 100mgPb/kg body weight) and three control groups exposed to the same concentrations of sodium acetate (25, 50, 100mgNa/kg body weight). The selected doses are in agreement with a previous study on a different amphibian species using lead acetate [25]. All the administered doses were determined previously as sublethal for some adult anuran toads being equivalent to 1/89–1/9 of the five days -LD50 [26]. Experimental toads received one single injection of the lead acetate or sodium acetate in the dorsal lymph sac. Toads were kept unfed during both the acclimation and experimental periods. Two weeks after the injections, animals were double pithed and blood was collected in heparinized polyethylene tubes [27]. Blood parameters and genotoxicity techniques were performed as mentioned below.

2.2. Tissues Analysis

Liver and muscles were transported in ice container to the laboratory for chemical analysis. They were washed with tap water (previously analyzed for Pb and Cd) followed by bi-distilled water. One gram portions of tissues were digested according to [28, 29]. Concentrated nitric acid HNO_3 (10 ml; trace metal grade), hydrogen peroxide H_2O_2 (30%) and hydrochloric acid Hcl were used to extract metal from tissues.

Lead concentration in tissues was determined by a Perkin–Elmer spectrometer. Pb concentration was calculated in µg/g wet weight for tissue,

$$\text{Tissue concentration}=\frac{\text{Reading}\times\text{dilution}}{\text{Weight (g)}}$$

2.3. Haematological Analysis

Blood samples from *Amietophrynus regularis* were collected from heart ventricle after anesthetized by ether. Sample was freshly collected in a small glass tubes containing 1 mg of disodium salt of EDTA (Ethylene diamine tetraacetatic acid) [24, 30, 31] as anticoagulant. The whole blood was used for the estimation of haemoglobin concentration (Hb), red blood cells count (RBCs), packed cell volume (PCV), and White blood cells count (WBCs). Red blood cells and white blood cells were counted manually using haemocytometer [24, 32, 33]. Haemoglobin concentration (Hb) was detected using Sahli device [34, 35]. Microhaematocrit pipettes of 1.1 mm in diameter and 75 mm

in length were utilized in detecting packed cell volume (PCV) [36]. For differential leukocytes count, [37] Lieshman stain was used.

2.4. Micronuclei Test and Nuclear Lesions

To asses DNA damage, MN and NL tests were performed according to [38, 39]. Nuclear abnormalities were classified according to [40, 41]. Binucleated (BN) erythrocytes are those with nearly two equal-sized nuclei. Blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than the blebbed nuclei which could have several lobes were classified as lobed nuclei. Nuclei with vacuoles and appreciable depth into a nucleus that does not contain nuclear material were recorded as notched nuclei. Kidney- and heart shaped nuclei both present an appreciable depth in to a nucleus that does not contain nuclear material. Irregular-shaped nuclei are those with irregular shape. Blood samples were smeared on cleaned glass microscope slides. After fixation in pure methanol for 20 min, slides were air–dried and then stained with 10% Giemsa solution. From each animal, 1000 cells were scored under 1000X magnification to determine the frequencies of micronucleus (MN), binuclei (BN), notched, lobed, heart shaped, kidney shaped, irregular shaped and blebbed nuclei. Only the cells clearly isolated from the surrounding cells were scored.

2.5. Statistical Analysis

Analysis of variance on SPSS software package program (Version 17) was used to test the current data. In the case of significant differences, the Multiple Range Comparisons (Least Significant Difference; LSD) was selected from Post Hoc window on the same statistical package to detect the significant difference between means. Paired sample test was applied to evaluate the significant differences between means of some pairs of the current data.

Probability values ≤0.05 and ≤0.01 were defined as significant throughout the current work. However, the values > 0.05 were considered non–significant. Probability values between 0.05 and 0.01 (both are included) were evaluated as significant. Statistically insignificant, significant and highly significant outputs were accompanied by symbols [^{NS}, ^{a} and ^{aa}] respectively. A linear regression was used to describe the relationship between Pb distribution in tissues and hematology, in addition to predict the relation equations between metal concentrations in tissues and the appearance of micronucleated RBCs and nuclear lesions.

Regression model was expressed using the following equation:

$$Y=a+b\,X$$

Where
X ⟹ is the explanatory variable
Y ⟹ is the dependent variable.
B ⟹ is the slope of the line.
A ⟹ is the intercept (the value of y when X=0).

3. Results

3.1. Bioaccumulation of Pb in Tissues

The present work resulted remarkable increase in Pb accumulation in both, liver and muscles with the increasing of exposure concentration compared to the control groups (Table 1). Non-significant differences were observed in the level of lead in the control groups either in liver (3.27±0.52-3.33±0.62µg/gm) or in muscles (2.07±0.67-2.71±0.67ug/gm). Oppositely, Bioaccumulation of Pb in liver was found to be increased from (6.82±0.65 µg/gm) to (11.07±0.79 µg/gm) responding to the increase in Pb doses from (25mg/kg) to 100mg/kg). In the same way, the bioaccumulation in muscles was responded to the lead doses and increased from (5.54±0.72 µg/gm) to (7.46±0.60 µg/gm). Bioaccumulation of Pb was greater in liver than muscles (Table 2 and Figure 1).

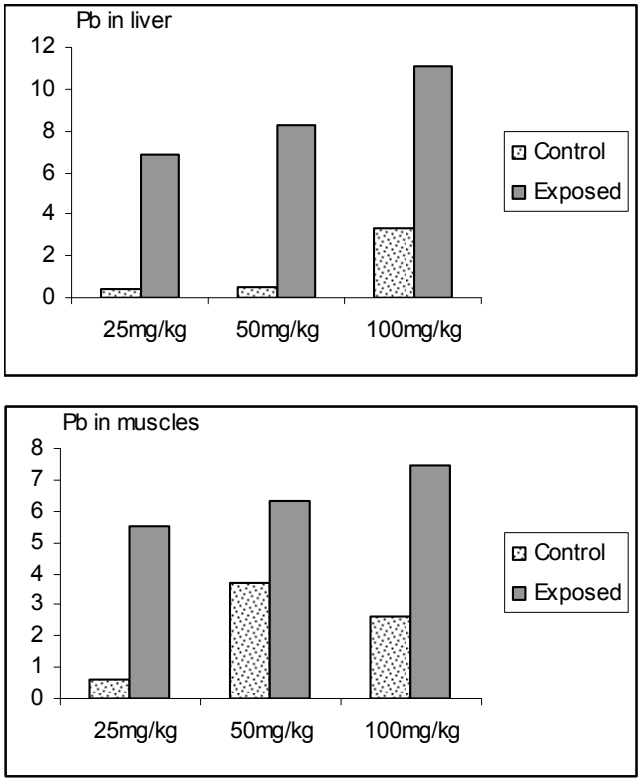

Figure 1. *Lead concentration in tissues of control and Pb exposed Amietophrynus regularis.*

Table 1. *Lead concentration in the control (Na act. treated) and Pb -exposed Amietophrynus regularis groups.*

Na act. treated	Control	
	Liver	Muscles
25mg/kg Na act.	3.28±0.47	2.07±0.67
50mg/kg Na act.	3.27±0.52	2.71±0.67
100mg/kg Na act.	3.33±0.62	2.61±0.69
Pb-exposed	Exposure	
	Liver	Muscles
25mg/kg Pb act.	6.82±0.65	5.54±0.72
50mg/kg Pb act.	8.31±0.62	6.29±0.83
100mg/kg Pb act.	11.07±0.79	7.46±0.60

Table 2. *Paired sample test showing differences in Pb bioconcentration in tissues (Liver – Muscle) of Amietophrynus regularis exposed to Pb for 14 ds.*

	Paired Differences					t	df	Sig.
	Mean	SD	SD Mean	95% CID				
				Lower	Upper			
Liver Pb –Muscle Pb	2.30	1.48	.38	1.50	3.12	6.07	14	.000

3.2. Haematological Parameters

Some blood parameters have shown significant differences among different doses (Table 3). For example, RBCs count, was observed to be decreased as Pb dose increase ($p<0.05$). Although haemoglobin exhibited no significant differences between 25 and 50 mg/ kg Pb concentration, it exhibited high significant differences between 25 & 100 mg/ kg Pb ($p<0.01$), 50 & 100mg / kg Pb ($p<0.05$), and between control and exposed groups as well. Haematocrit (packed cells volume) PCV showed significant differences among all the treated groups and between the treated and control groups.

To predict the effect on Pb level in tissues on some major blood parameters, stepwise regression has been conducted (Tables 4 and 5):

- Decline in RBCs count could be predicted from the linear combination between Pb in liver and counted RBCs ($p<0.01$) and Pb in muscles and counted RBCs ($p<0.05$). The regression model can be summarized as follow:

RBCs count=461640.319-(9037.955×Pb liver).

RBCs count=472895.920-(14032.543×Pb muscles).

- Decline in Hb value could be predicted from the linear combination between Pb in liver and Hb value ($p=0.01$) and Pb in muscles and Hb value ($p<0.01$). The regression model can be summarized as follow:

Hb value=9.515-(0.283×Pb liver).

Hb value=10.680-(0.566×Pb muscles).

- Elevation in WBCs count could be predicted from the linear combination between Pb in liver and counted WBCs ($p<0.01$) and Pb in muscles and counted WBCs ($p<0.01$). The regression model can be summarized as follow:

WBCs count=5247+ (296×Pb liver).

WBCs count=5030+ (436×Pb muscles).

Table 3. *LSD test showing the blood parameters of toads exposed to different doses of Pb in relevant to control groups.*

Parameters	Control&25Pb	Control&50Pb	Control&100Pb	25 Pb&50Pb	25Pb&100Pb	50Pb&100Pb
RBCs	412000^{aa}	444000^{aa}	462000^{aa}	32000^{aa}	50000.0^{aa}	18000.0^{NS}
Hb	7.58^{aa}	6.19^{aa}	7.35^{aa}	0.23^{NS}	1.39^{aa}	1.17^{a}
PCV	32.25^{aa}	21.6^{aa}	24^{aa}	0.024^{aa}	0.048^{aa}	0.024^{aa}
WBCs	-3120^{aa}	-3640^{aa}	-4500^{aa}	-0.52^{a}	-1.38^{aa}	-0.86^{aa}

a: The mean difference is significant at the 0.05 levels
aa: The mean difference is significant at the 0.01 levels
NS: The mean difference is not significant

Table 4. *Linear regression between liver Pb and main blood parameters of Pb -exposed animals.*

			Uns. Coft.[2]		St. Coft.[1]		
Dent. Var.	Selected variable	R	B	S. Err	B	T	Sig.
RBCs	Constant	.65	461640.319	26087.62	-0.65	17.69	0
	Pb liver		-9037.955	2919.45		-3.09	0
Hb	Constant	.61	9.515	0.907	-0.61	10.49	0
	Pb liver		-0.283	0.102		-2.78	0.01
WBCs	Constant	.84	5.247	0.456	0.84	11.49	0
	Pb liver		0.296	0.051		5.79	0

[1]StandardizedCoefficients
[2]UnstandardizedCoefficients

Table 5. *Linear regression between muscles Pb and main blood parameters of Pb -exposed animals.*

			Uns. Coft.[2]		St. Coft.[1]		
Dent. Var.	Selected variable	R	B	S. Err	B	T	Sig.
RBCs	Constant	.55	472895.92	38099.55	-0.55	12.41	0
	Pb muscle		-14032.54	5851.57		-2.39	0.032
Hb	Constant	.67	10.68	1.13	-0.67	9.42	0
	Pb muscle		-0.566	0. 174		-3.25	0.006
WBCs	Constant	.68	5.030	0.838	0.68	6	0
	Pb muscle		0.436.	0.129		3.38	0.005

[1]StandardizedCoefficients
[2]UnstandardizedCoefficients

3.3. MN& NL Tests

Beside MN, nine other nuclear lesions were detected in the blood of the exposed toads (Table 6 and Figure 2). Control groups exhibited the lowest means of the scored RBCs abnormalities. Irregular shaped nuclei (ranged between 600±.547 in control and 12.400±2.07 in 100mg/kg Pb exposed groups) represented the higher frequencies compared to other nuclear lesions. The lowest NL frequency was heart shaped nuclei.

Furthermore, LSD analysis has summarized the significance degrees between induced RBCs abnormalities in relevant to Pb exposed groups (Table 7 and Figure 2):

Micronucleated RBCs, binucleated RBCs, lobed nuclei, irregular shaped nuclei and notched nuclei were significantly higher in the exposed groups compared to the control ones. Also, their frequencies increased significantly with the increasing of lead doses. Heart and kidney-shaped nuclei of *Amietophrynus regularis* exhibited significant differences between control & 100mg/kg Pb and between 25mg/kg Pb &100mg/kg Pb only. The mean of blebbed nuclei was significantly higher in the blood of toad exposed to 50mg/kg Pb ($p<0.05$) and 100mg/kg Pb ($p<0.01$) compared to the control ones. Also, a significant elevation was recorded with the increasing of lead doses.

Totally, induction of micronucleated, binucleated RBCs and erythrocytic abnormalities were found to be positively increased (correlated) with the increase in Pb in liver (excluded kidney shaped nuclei) and muscles (excluded heart shaped nuclei ($0.05\geq p\leq 0.01$) (Table 8).

Table 6. *Means ± SD of micronucleated and nuclear lesions (‰) of in the blood of the control and 25, 50 and 100 mg/l Pb exposed groups.*

Parameters	Control	25mg/kg Pb	50mg/kg Pb	100mg/kg Pb
MN	.200±.447	4.000±1.58	6.600±1.81	9.400±2.07
BN	.200±.447	2.600±.54	5.800±1.09	11.400±2.07
Lobe-shaped	.200±.447	3.800±.83	5.800±.83	8.800±1.48
Heart-shaped	0	0	.600±.54	.800±.83
Kidney-shaped	0	.600±.54	1.600±.89	2.200±.83
Irregular shape	.600±.547	5.200±1.48	8.600±1.14	12.400±2.07
Notched N	0	1.000±0.70	2.200±.83	4.000±1
Blebbed N	0	.600±.54	1.600±1.67	3.200±1.30

Table 7. *LSD multiple comparison testing the significant of micronuclei and nuclear lesions (‰) in the blood of Amietophrynus regular is exposed to different doses of Pb in relevant to control groups.*

Parameters	Control&25Pb	Control&50Pb	Control&100Pb	25 Pb&50Pb	25Pb&100Pb	50Pb&100Pb
MN	-3.8^{aa}	-6.399^{aa}	-9.200^{aa}	-2.599^{a}	-5.4^{aa}	-2.80^{aa}
BN	-2.4^{aa}	-5.6^{aa}	-11.2^{aa}	-3.199^{aa}	-8.8^{aa}	-5.60^{aa}
Lobe-shaped	-3.56^{aa}	-5.6^{aa}	-8.60^{aa}	-2.0^{aa}	-5.00^{aa}	-3.00^{aa}
Heart-shaped	0.0^{NS}	-0.6^{NS}	-0.8^{a}	-0.6^{NS}	-0.8^{a}	-0.20^{NS}
Kidney-shaped	-0.6^{NS}	-1.6^{aa}	-2.2^{aa}	-1.0^{a}	-1.6^{aa}	-0.6^{NS}
Irregular shape	-4.60^{aa}	-8.0^{aa}	-11.8^{aa}	-3.399^{aa}	-7.2^{aa}	-3.800^{aa}
Notched N	-1.0^{a}	-2.2^{aa}	-4.0^{aa}	-1.20^{a}	-3.0^{aa}	-1.8^{aa}
Blebbed N	-0.6^{NS}	-1.6^{a}	-3.2^{aa}	-1.0^{NS}	-2.6^{aa}	-1.6^{a}

Table 8. *Correlation coefficients between Pb concentration in tissues and (MN& NL) of Amietophrynus regularis.*

Parameter	Pb in liver	Pb in muscles
		($r^{Sig.}$)
MN	$.62^{a}$	$.65^{aa}$
BN	$.96^{aa}$	$.69^{aa}$
Lobed-shaped	$.91^{aa}$	$.66^{aa}$
Heart-shaped	$.67^{aa}$	$.25^{NS}$
Kidney-shaped	$.47^{NS}$	$.69^{aa}$
Irregular shape	$.80^{aa}$	$.84^{aa}$
Notched N	$.76^{aa}$	$.63^{a}$
Blebbed N	$.61^{a}$	$.59^{a}$

a: The mean difference is significant at the 0.05 levels
aa: The mean difference is significant at the 0.01 levels
NS: The mean difference is not significant

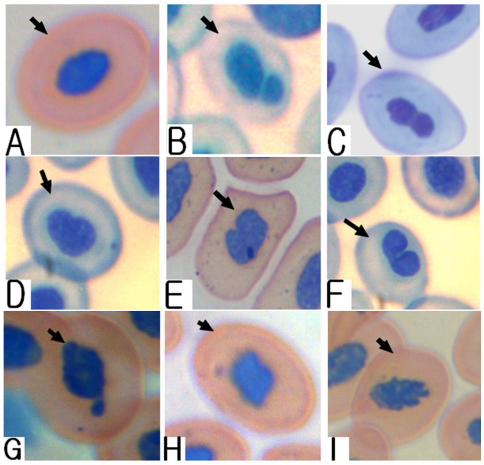

Figure 2. *Normal red blood cells (A) of Amietophrynus regularis and different types of nuclear abnormalities and lesions. Micronucleated RBCs (B), binucleated RBCs (C), RBCs with heart shaped nucleus (D) and kidney shaped nucleus (E). RBCs with notched nucleus (F), blebbed (G) and irregular shaped (H). Erythrocytes with lobed nuclei (I). Giemsa stain, X1000.*

4. Discussion

Lead is released into the atmosphere from natural and anthropogenic sources. Natural emissions are from wind resuspension and from sea salt, volcanoes, forest fires and biogenic sources [42]. According to Nriagu [42], these emissions are not entirely natural but contain some contributions from historical depositions of anthropogenic lead. Major anthropogenic emission sources of lead on a global scale include the combustion of fossil fuels from, for example, traffic, nonferrous metal production and iron and steel production. Some contributions are also made by cement production and waste disposal [43]. Emissions, house dust and paints must be taken into consideration. Heavy metals can accumulate in the top soil from atmospheric deposition by sedimentation, impaction and interception [44]. The variations in the levels of lead in roadside soil are frequently attributed to traffic density [45]. Pb was known as one of the common toxic metals that display increases in body concentrations as exposure levels increase [46].

Either in the liver or muscle, it has been observed that an increase in the administered dose was associated with significant increase in the bioaccumulation of lead. Experimentally, several studies dealt with metal exposure demonstrated that metal accumulation in tissue was dosed metal concentrations – dependant (e.g. *Bufo maculates* [47, 48] *Hoplobatrachus occipitalis maculates* [49]; *Hoplobatrachus occipitalis* and *Bufo maculates maculates* [50, 51]. The present result confirmed the ability of *Amietophrynus regularis* to take up and accumulate heavy metals from their ambient habitat. Such result was strongly supported by Ezemonye [50] in flat backed toad, *Bufo maculates maculates.* Also, the results of this study agreed with those of James et al., [52] who observed that fish exposed to sub lethal levels of lead produced dose – dependent significant increases in the concentration of lead in the liver and muscle of *Oreochromis mossambicus*.

The impact of heavy metals on blood parameters of aquatic animals has been reported to introduce adverse effects. Data on lead toxicity on lower vertebrates and in particular on adult amphibians is not frequent [45]. Accordingly, in the present work Pb was selected to evaluate metal impact on haematology of *Amietophrynus regularis*. The results of the present investigation showed that the lead treatment inflicted a drastic reduction in the means of RBCs and haemoglobin in addition to remarkable increase in WBCs, impairing the major blood parameters in this investigation. Correlation analysis has demonstrated a negative effect of Pb accumulation in liver and muscles on RBCs count, haemoglobin, haematocrit and mean cell volume ($0.05>p\leq0.01$). Oppositely, Pb in muscles and liver exhibited a positive effect in WBCs count.

There are relatively few studies concerning toxic effects of lead in adult amphibians [36, 53], but some lab studies have shown alterations in the synthesis of the haeme group through inhibition of the activity of the enzyme delta-aminolevulinic acid dehydratase (δ- ALAD) affecting the synthesis of haemoglobin [54, 55]. Exposure of adult anurans to lead exhibited an increase in erythrocytes' osmotic fragility [56], decrease of total and differential leukocyte counts [57], increase in levels of erythrocyte protoporphyrin [54], micronucleus induction and nuclear abnormalities [58].

In accordance with the present work, blood parameters of the catfish *Clarias batrachus* were deviated from control groups after exposure to different mercury concentrations [59]. They resulted gradual decrease of RBCs count and haemoglobin in addition to gradual increase in WBCs count compared to control groups. Exposure of freshwater fish, *Channa punctatus* to copper showed a significant decrease in the haemoglobin (Hb) content, red blood cells (RBC) and packed cell volume (PCV) compared to the control.

Amietophrynus regularis exposed to lead has exhibited significant decrease in haemoglobin, indicating that lead could cause anaemia. The observed reduction in the RBCs count, haemoglobin and haematocrit values may be attributed to the decreased rate of production of red blood cells or an increased loss of these cells. Anaemia could be attributed to impaired erythropoiesis due to the direct effect of heavy metals on hematopoietic centers (kidney /spleen). On the other hand, white blood cells play major role in the defense mechanism of animal. They consist of granulocytes, monocytes, lymphocytes and thrombocytes [60]. In the present investigation, leukocyte concentration showed greater and quite different pattern of change with the effect of lead when compared with the leucocytes levels of the control toads. Blood of all experimental groups contained higher concentrations of leucocytes than those of controls. Increased WBC counts in *Aureus oreochromis* after mercury exposure was observed by Allen [61]. The increase in the number of WBCs observed in the present study may be attributed to the stimulation of immune system in response to tissue damage caused by lead, in agreement with several previous reports. For example, [62, 63] reported an increase in WBCs. Total WBCs count increased in *Tinca tinca* exposed to lethal and sublethal treatments with mercury [32]. Also, Oliveira et al., [64] observed increase in the leucocytes number in fish *Hoplias malabricus* exposed to subchronic and dietary doses of methyl mercury. Singh and Nath [65] stated that high white blood cell counts indicate damage due to infection of body tissues caused by metals, severe physical stress, and leukemia as well. Some of the most common causes of heavy metal toxicity are inflammatory lesions associated with tissue damage, anaemia and neoaplasia. Further, an increase in fibrinogen or serum globulins or a decrease in serum albumin may be as a result of metals impact [65].

In this study, higher incidences of MN and NL were found in the blood of toad exposed to lead doses. Such frequencies were significantly elevated with the increasing lead doses. The frequencies of MN and NL were significantly higher in blood erythrocytes of toad exposed to the highest lead dose. The lowest level of genotoxicity was observed in the blood of control toad. In the present work a positive correlation was demonstrated between the investigated heavy metals in

tissues and the induction of micronucleated RBCs and nuclear abnormalities in *Amietophrynus regularis*. There was, therefore, a direct relation between bioaccumulation of heavy metals and the formation of micronucleated RBCs.

These findings agree with Normann et al., [22], who clarified the presence of strong relation between heavy metals exposure and the appearance of the micronuclei in the blood of the catfish *Hypostomus plecotomus*.

In accordance with the present results, the induction of MN was significantly increased as Pb doses increased in suckling rats [66]. The present *means of MN in the blood of Amietophrynus regularis* (4.000±1.581‰ - 9.400±2.073‰) were higher than those in the blood of *Odontophrynus cordobae* and *Rhinella arenarum* [31].

The results of this study confirm the usefulness of the erythrocyte MN and NL as powerful monitoring tools for detecting genotoxic agents in aquatic and terrestrial environment. Background levels of MN and NL incidences were observed in the blood of all the control and treated toads. A positive correlation was recorded in this investigation between MN and NL. In accordance with these results, Osman and Harabawy [67] observed a correlation between the frequencies of MN and NL, suggesting the importance for recording this anomaly in order to enhance the importance of data obtained by the MN test. Moreover, a positive and significant relationship between MN and other NL was found [68, 69, 23], indicating that NL formations in erythrocytes is a suitable complementary assay for genotoxicity assessment in fish.

One of the mechanisms responsible for DNA synthesis impairment is the well-known oxidative stress [70]. Recent studies indicate that transition metals act as catalysts in the oxidative reactions of biological macromolecules therefore the toxicities associated with these metals might be due to oxidative tissue damage. Cells under oxidative stress display various dysfunctions due to lesions caused by ROS to lipids, proteins and DNA.

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