

DNA damage in hemocytes of *Schistocerca gregaria* (Orthoptera: Acrididae) exposed to contaminated food with cadmium and lead

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ABSTRACT

We measured in a comet assay the damage of DNA in the hemocytes of various stages of the grasshopper *Schistocerca gregaria* after exposing them to various doses of Cd and Pb in the food. The mechanisms of Cd and Pb toxicity for grasshopper are discussed. The accumulation of heavy metals and stage of the insect may play important roles in causing the DNA damage. *S. gregaria* may be considered a valuable bio-indicator for evaluation the genotoxicity of environmental pollutants.

Keywords: Comet Assay; Heavy Metals (Cd, Pb); DNA Damage; *Schistocerca gregaria*

1. INTRODUCTION

Heavy metals are among the most problematic causes of water, soil and plant pollution. Genetic and biochemical effects of pollutants on organisms are important in establishing species as bioindicators for environmental hazards [1,2]. Heavy metals have been found to induce genotoxic effects in chironomids which are used as a good bioindicator group for aquatic pollution [3]. Terrestrial insects that develop in the soil are also exposed directly to metal ions present in the soil. Grasshopper species may provide good systems to evaluate the mutagenic effects of some environmental contaminants [4-6].

Cadmium and lead are widespread and dangerous heavy metals that are released into the environment from many sources. Their accumulation in the soils can become dangerous to all kinds of organisms, including plants and human life, causing many genotoxic effects [7-9]. They are highly toxic and have been recognized as poison and a probable carcinogen [10]. Clinically, they can adversely affect human health, especially the blood

and the renal system [11].

Changes in the cell genome caused by genotoxic agents leading to mutations and possibly tumor formation are some of the lethal or sub-lethal effects induced by a complex mixture of pollutants. Among recently used methods to identify DNA damage is the comet assay (SCGE-single cell gel electrophoresis). The comet assay provides a rapid, sensitive, and inexpensive method to detect DNA strand breaks in individual eukaryotic cells [12]. Despite some difficulties in obtaining cell/nuclei suspension, this method has been used to detect and evaluate DNA damage caused by double strand breaks, single strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. It has been successfully applied to cells of various animal groups [13]. Only a few studies have been reported on DNA damage in insects, including *D. melanogaster* [14], and in the weevil *Curculio sikkimensis* [15], and in grasshoppers *Chorthippus brunneus* [16].

The aim of the present work was to determine the genotoxic effect of cadmium and lead on the locust *S. gregaria* and to evaluate its potential as a biomonitor for detecting a heavy-metal polluted environment.

2. MATERIALS AND METHODS

2.1. Colonization of *S. Gregaria*

Locusts were reared in wooden cages at $32 \pm 2^\circ\text{C}$, 50-60% RH and 16 hrs day light in our Entomology Department since about 10 years ago. A daily supply of fresh grass, clover plant was supplied to the locusts. Packed moist sterilized sand in suitable glass containers about 7 cm in diameter and 10 cm deep were prepared for egg-laying.

2.2. Heavy Metals Treatment and Sample Preparation for Alkaline Single Cell Gel (SCG) Assay

Living individuals of *S. gregaria* of the 4th, 5th instars,

and newly emerged (NEA)(4 days old) and mature (15 days old) adult (MA), fed on treated clover (their stems were previously immersed for 24 hrs in distilled water containing 25 mg and 50 mg/L of CdCl₂ and PbCl₂, to allow the clover to absorb contaminated water) or on untreated clover, were collected from their respective cage. Haemolymph samples were withdrawn from the collected insects by means of micropipettes at incision made near the 3rd coxae. Five insects were used for each sample.

2.3. Detection of DNA Damage Using Alkaline SCG Assay

Biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking with the single cell were done according to the alkaline (pH 13) SCG assay, and developed [17].

The alkaline version of comet assay was used to analyze the level of DNA damage in the hemocytes of *S. gregaria* to estimate the genotoxic effects of Cd²⁺ and Pb²⁺. 20 μL of hemolymph from the pool of 5 insects were centrifuged at 1000 rpm for 10 min. Isolated hemocytes were immediately suspended in cooled 50 μL Ringer solution and kept on ice, in darkness. 10 μL of isolated cells were mixed with 90 μL of 0.75% low melting point agarose (LMPA), and placed on a microscope slides, pre-coated with 1.5% normal melting point agarose (NMA). A cover slip was added, and the slides were immediately placed on ice. After agarose solidified, cover slips were removed, and the slides were immersed in a lyses buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.25 M NaOH, 1% TritonX-100, and 10% dimethylsulfoxide (DMSO), pH 10.0) for 2h at 4°C. After the lysis, the slides were placed in a horizontal gel electrophoresis tank and DNA was allowed to unwind for 20 min in electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13). Electrophoresis was carried out at 21 V and 270 mA, at 4°C, for 15 min. Then the slides were neutralized in 0.4 M Tris-HCl (pH 7.4), fixed with methanol and allowed to dry overnight at room temperature before staining with ethidium bromide (2 μg/mL). Comets were analyzed with Axio fluorescence microscope (Carl Zeiss, Germany) with an excitation filter of 524 nm and a barrier filter of 605 nm. Three replicates were prepared and each of them consisted of a pool of 5 individuals.

2.4. Evaluation of DNA Damage

DNA damage was visualized with fluorochrome stain of DNA with the fluorescent microscope and a 40X objective (depending on the size of the cells being scored). A Komet analysis system 4.0 developed by Kinetic Imaging, LTD (Liverpool, UK) linked to a CCD camera was used to measure the length of DNA migration (Tail length) (TL), and the percentage of migrated DNA (DNA %). To

distinguish between populations of cells differing in size nuclear diameter was measured. Finally, the program calculated tail moment. 50-100 randomly selected cells are analyzed per sample (at least 25 cells per slide and 3 slide per treatment were evaluated).

Statistical analysis for data was done using ANOVA and T-test analysis, based on a minimum of 4 individual insects per group. In addition, numbers of cells were analyzed to exhibit values greater than the 95 or 99% confidence limits for the distribution of control data.

3. RESULTS

3.1. Comet Assay of DNA Damage

The typical DNA damage of haemolymph cells of *S. gregaria* exposed to low and high concentrations of cadmium chloride (CdCl₂) and lead chloride (PbCl₂) in the food can be seen in **Figure 1**. The haemolymph cells of the control showed almost rounded nuclei (**Figure 1(a)**). In the haemolymph cells of the heavy metals contaminated insects, the nuclei with a clear tail like extension were observed indicating that the haemolymph cells of the insect were damaged and DNA strand breaks had occurred (**Figure 1**).

The typical DNA comet for hemocytes of *S. gregaria* showed illustration of rounded nuclei of control and maximum length of tail formed and migration of DNA in this tail under the effect of contamination with different concentrations of CdCl₂ and PbCl₂ (**Figure 1**).

The DNA damage of the hemocytes of different stages of *S. gregaria* fed on clover exposed to low and high concentrations (25 and 50 mg/L) of CdCl₂ and PbCl₂ was analyzed quantitatively by comet assay and expressed as tail length (TL), DNA % and tail moment (TM) (**Table 1**, and **Figures 2** and **3**). It was found that low concentration

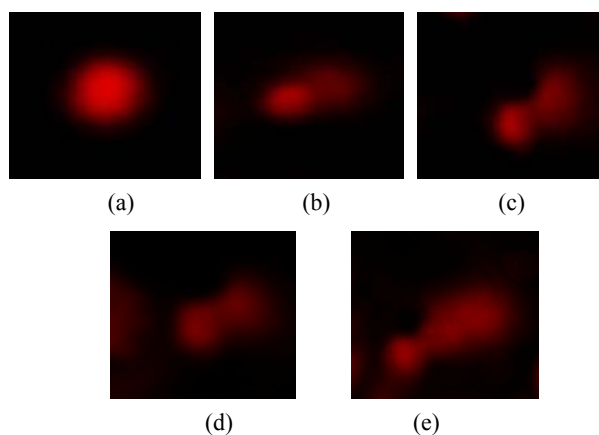


Figure 1. Typical DNA comet from haemocytes of 4th instar *S. gregaria*. (a) Control; (b,c) Low and high concentrations of CdCl₂ respectively; (d,e) Low and high concentrations of PbCl₂, respectively.

Table 1. Detection of DNA damage by the comet assay, assessed as tail moment (TM) in hemocytes of 4th, 5th, NEA, and MA of *S. gregaria* exposed *in vivo* to CdCl₂ and PbCl₂, at different doses in the food.

Agent / Dose	4 th instar	5 th instar	NEA	MA
Control	0.028 ± 4.4 × 10 ⁻³	0.095 ± 5.8 × 10 ⁻³	0.009 ± 2.3 × 10 ⁻³	0.073 ± 3.5 × 10 ⁻³
CdCl ₂ (mg/L)				
25	0.092 ± 0.02*	0.16 ± 0.017*	0.04 ± 3.5 × 10 ⁻⁴ *	0.29 ± 0.0133*
50	0.057 ± 3.3 × 10 ⁻³ *	0.07 ± 6.9 × 10 ⁻³	0.08 ± 3.9 × 10 ⁻³ *	0.45 ± 0.03*
PbCl ₂ (mg/L)				
25	0.081 ± 0.022*	0.124 ± 0.012	0.05 ± 5.8 × 10 ⁻³ *	0.32 ± 3.5 × 10 ⁻³ *
50	0.7 ± 8.8 × 10 ⁻³ *	0.11 ± 0.017	0.021 ± 8.8 × 10 ⁻³ *	0.44 ± 0.035*

Significant at **P* < 0.05; in all cases significance was tested with respect to 0 (control) using t-test, (N = 3). Values are expressed as means ± S.E.

of CdCl₂ (25 mg/L) caused a significant increase in the values of TM in the hemocytes of different stages. While, the high concentration (50 mg/L) of CdCl₂ caused a lower significance increase in TM in the 4th instar, somewhat insignificant increase or decrease in 5th instar and led to a significant higher increase of these values in the adult stage (NEA and MA). Low and high concentrations of PbCl₂ caused a significant increase in TM, generally in all developmental stages with few insignificant changes. The effect of the PbCl₂ concentration was not clear as in CdCl₂ (Table 1).

The damage of hemocyte DNA expressed as TL and DNA% under the effect of different concentrations of CdCl₂ and PbCl₂, analyzed by the comet assay (Figures 2 and 3). It was found that 25 mg/L of CdCl₂ caused a significant increase in the values of TL, DNA% in the hemocytes of different stages. The high concentration (50 mg/L) of CdCl₂ caused a lower significance increase in TL in the 4th and 5th instar. Low and high concentrations of PbCl₂ caused a significant increase in TL, and DNA%. The prominent increase in the values of TL and DNA% in response to contamination with Cd and Pb was observed in the mature adult stage (MA). The dose concentration of Cd and Pb had insignificant effect on the values of TL and DNA % (Figures 2 and 3).

The analysis of variance of the two factors (stage and heavy metal concentrations) showed that, the stage of the insect had a clear significant effect on the DNA damage (TL, DNA % and TM). A less significant effect of the dose (concentration of heavy metals) was observed (Table 2).

3. DISCUSSIONS

In the present study, the treated clover exposed to CdCl₂ and PbCl₂, at doses of 10 and 20 mg/L, contained 10 and 20 µg/g plant tissues, respectively to each dose (data not presented). The exposure of *S. gregaria* to Cd and Pb in

the food caused an increase in damage (expressed as TL, TM, and DNA%) of DNA of hemocytes. However, the obtained data were sometimes ambiguous; for instance, the TL was not proportional to the Cd dose in the 4th and 5th instars but was true in the NEA and MA (Figure 2). The available data from the literature are from assays on cell cultures (mostly human or rat lymphocytes) and many of them concerning genotoxicity of Cd, As, Pb, and Hg [13,18,19].

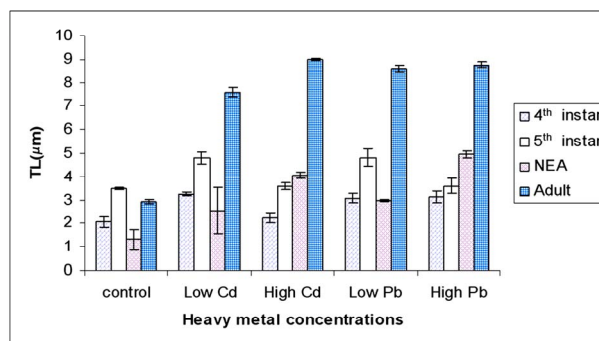


Figure 2. Comet TL data of hemocytes from different instars of *S. gregaria* exposed to food with low (25 mg/L) and high concentration (50 mg/L) of Cd and Pb.

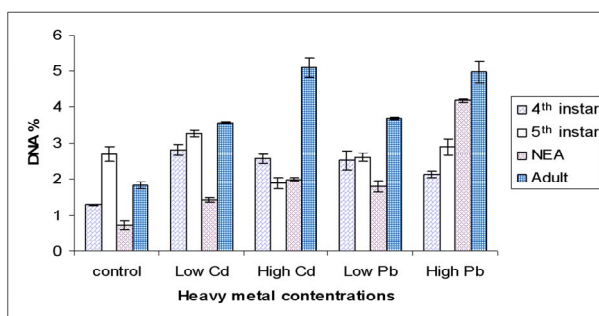


Figure 3. Comet DNA% data of hemocytes from different instars of *S. gregaria* exposed to food with low (25 mg/L) and high concentration (50 mg/L) of Cd and Pb.

Table 2. Analysis of variance (Two Way ANOVA) for tail length (TL), DNA %, and tail moment (TM) in *S. gregaria* with heavy metal treatment as categorical factors.

Source of Variation	TL			DNA %		TM	
	Df	F	P	F	P	F	P
Stage (1)	3	181.3	0.00001	133.6	0.00001	358.3	0.00001
Heavy metal Concentration (2)	4	44.1	0.00001	78.1	0.00001	73.9	0.00001
Interaction 1 × 2	12	12.4	0.00001	29.7	0.00001	36.1	0.00001

The no increased or even decreased DNA migration using comet assay may reflect the cells with DNA cross-linking lesions [19,20]. The higher DNA damage in the mature adults with respect to the long period of exposure to heavy metals (**Figures 2 and 3**) reflects the absence of repair mechanism in this insect at the used concentrations of CdCl₂ and PbCl₂.

3.1. Cadmium Genotoxicity

Exposure of *S. gregaria* to Cd in their food leads to accumulation of the metal in the insect body of 10, 20, 10, and zero µg/g insect body in the 4th, 5th, NEA, and MA (data not presented data). The accumulation factors of heavy metals in grasshoppers were found in the order Cd > Hg > Pb indicating a greater affinity for Cd accumulation. With the growth of muscle tissues and fat bodies during post-embryonic development (nymph to adult), the concentration of Cd was found to be steadily increasing [21]. It has been suggested that the molecular mechanism for the genotoxicity of cadmium may involve either indirect or direct interaction of with DNA [22], such as DNA strand breaks [10], DNA protein-cross linking [23], Oxidative DNA damage [24], enhanced proliferation, depressed apoptosis and inhibition of DNA repair [24-26].

Many metals, including cadmium, in biological systems form complexes with nucleophilic ligands of target molecules [27]. The affinity of cadmium is higher for biomolecules containing more than one binding site such as metallothionein [7]. Another factor of cadmium toxicity is that it replaces zinc in enzymes, thereby inhibiting their activity [28]. Some insects as *D. melanogaster* have tolerance to heavy metals [27], and their natural populations differed in amplification of the metallothionein gene [29]. By binding to plasma membrane receptors, cadmium stimulates release of calcium from intracellular storage sites [7]. Moreover elevated cadmium levels may inhibit Ca-ATPase working in the plasma and endoplasmic reticulum membranes, leading to disturbance of calcium homeostasis [7,25]. Also, it inhibits DNA repair enzymes, such as DNA polymerases by binding to nucleic acids and chromatin [25].

3.2. Lead Genotoxicity

The exposure of grasshoppers to Pb in the food caused an increase of DNA damage in haemolymph cells. The increase of TL values was proportional to the Pb dose in

the food in 4th nymphal instars, newly emerged, and mature adults but not proportional in 5th instars (**Figure 2**). This may be due to high retention of metals in the 5th instar as compared to the other stages. Dietary factors greatly influence lead retention. Several mechanisms could intervene in these effects. Low dietary calcium and lead-binding proteins at the sites of absorption [30] influence lead retention, because lead interferes with the regulation of calcium metabolism [31]. An interaction of lead and calcium can occur on the sites of toxic action by binding to phosphate groups, or by interfering with uptake in organelles etc.

There are several mechanisms how lead might interfere with repair process. Their ions may interfere with calcium regulated processes involved in the regulation of DNA replication and repair [32], induced genome damage includes DNA single-strand and double-strand breaks, DNA-DNA crosslinks, induction of reactive oxygen intermediates [33], and consequently acts as co-clastogens or co-mutagens [34].

The present work clearly shows that, the significant increase of genotoxicity in relation to the development of nymph to adult stage may be due to accumulation of heavy metals in the tissues and blood. This suggests that the DNA damage increased with Pb in blood. Likewise, a significant correlation was found between Pb accumulated in the blood and genotoxic effects in Pb exposed workers [35].

In conclusion, the genotoxicity of cadmium and lead in *S. gregaria* was very high in the mature adult stage; irrespective of the heavy metal dose and accumulation in the cells. So this may reflect the role played by *S. gregaria* as a valuable bioindicator for environmental genotoxic pollutants.

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