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Effects of Heavy Metals on the polytene chromosomes of *Chironomus* larvae

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Abstract

Chironomus larvae are widely used for toxicity testing. They have an immense response to heavy metals, and these effects may be chronic or acute. Chironomus larvae are characterized by the occurrence of polytene chromosomes in the salivary gland, which are formed due to repeated replication without division. Heavy metals are used in trace amounts in the metabolic activity of the larvae. However, excess exposure may lead to genotoxicity. In this study, the effects of lead acetate, ammonium dichromate, copper sulphate, and cadmium sulphate on the polytene chromosomes of Chironomus larvae were analysed. Additionally, survival time was examined after exposure to four heavy metals used in this study. This study shows that high concentrations of heavy metals beyond the concentration used for normal metabolic activity may lead to genotoxic effects and alter the structural and functional characteristics of the polytene chromosomes. Thus, Chironomus larvae have the potential to be used as bioindicator of heavy metal stress.

Key Words: *Chironomus* larva, heavy metal stress, polytene chromosomes

Introduction

The non-biting midge genus *Chironomus* belongs to the subfamily Chironomidae (Stur et al., 2020), and it has a number of cryptic species that can only be identified by specialists using the traits of their tremendous chromosomes. E.G. Balbiani published the first description of polytene chromosomes in 1881 (Zhimulev et al., 2009). The giant chromosomes, or polytene chromosomes, are typically found in many Dipteran (two-winged) flies such as *Drosophila, Chironomus,* and *Rhynchoscira* (Sumner et al., 2008). These are created when endo-reduplication or several rounds of DNA replication without cell division results in a large number of sister chromatids that continue to remain united (Stormo et al., 2017). The salivary gland of larvae is where polytene chromosomes are typically found, where several copies of the chromosomes allow for more powerful secretary activity. Simply said, each cell can now produce DNA at a considerably higher rate than a diploid cell with only two copies of each gene.

At interphase (mitosis and meiosis), polytene chromosomes exhibit unique thick and thin banding patterns. These patterns were initially employed in taxonomic identification and chromosomal mapping (Reddy et al., 2017).

The existence of several longitudinal strands termed Chromonemata, which give polytene chromosomes their huge size, gives rise to the word polytene (many stranded). They have a diameter of 20 µm and a length of roughly 0.5 mm (Callan, 2012). Dark bands and light bands (inter-bands) are the two types of bands found on polytene chromosomes (De et al., 1998). Nuclear stains produce mildly stained region in the inter-bands but deeply stained region in the dark bands. At specific moments, the polytene chromosomal bands grow to generate swellings known as a puff which is due to puff forming mechanism. The chromonemata coiling opens and extends out to form several loops in the puffy regions. The uncoiling of individual chromosomes results in the puffing. The puffs in a band show the location of actively translating genes. The puffs produce a lateral array of many loops. These loops are known as Balbiani rings in honour of the scientist who first identified them. They are made up of a few proteins, RNA, and DNA. The machinery for transcription and translation, including RNA polymerase and ribonucleoproteins, are located here (Berendes et al., 1968). Polytene chromosomes have a metabolic benefit because having several copies of a gene allows for high levels of gene expression, which increases the size of the cell nucleus and causes cell expansion (Frawley et al., 2015). Inter-bands support several processes such as nucleosome remodeling, origin localization structures, and connection with functional chromatin proteins. Their key roles involve serving as RNA polymerase II binding sites to commence replication and to begin nucleosome remodeling of short DNA fragments (Ramani et al., 2016).

According to Kiknadze et al. (1975), DNA strands replicated 10–11 times were found in the chromosomes that are polytene in the saliva producing gland of *Chironomus* larvae at phases 6-7 of the IVth instar. These minute structural rearrangements can be studied detail with the help of light microscopy. The salivary polytene chromosome have a distinct and well described band structure which permits detailed analysis of structural and functional observations (Michailova et al. 2002).

The cell needs several heavy metals for its regular metabolism. However, chronic toxicity occurs when metabolic limits are exceeded. Chronic toxicity refers to the ability of metals to affect metabolism for an extended length of time at concentrations above normal. Acute toxicity, on the other hand, is shown when heavy metals have a short-lived but significant impact on metabolism (Jaishankar et al., 2014). Lead is recognized as serious pollutant due to its toxicity, bioaccumulation, persistence, and extensive industrial use. Numerous cytogenetic impacts on the fresh water biota have been linked to lead acetate exposure. It has been demonstrated to have a negative impact on the "fitness" of a cell, including slowing down cell division and causing a variety of chromosomal abnormalities (Michailova et al., 2002). A precise cytogenetic test for the examination of chromosomal abnormalities is required to evaluate the genotoxicity of lead acetate. Environmental contamination and exposure to chemicals, including trace metals, under controlled conditions induces abnormal rearrangement and changes in the Balbiani ring structure of the salivary polytene chromosomes of *Chironomus* larva (Diez et al., 1990; Michailova et al., 1996, 1998). Chromium is a vital component of the diet of animals, mostly involved in the metabolism of carbohydrates and perhaps in the metabolism of lipid. (Michailova et al., 2001).

Hexavalent salt of chromium is mainly associated with the hazardous effect of chromium (Leonard & Auwerys, 1980). It rapidly transforms into a trivalent state and is extremely unstable and imbalanced. Ammonium dichromate has a genotoxic impact that can be shown when the presence of heavy metals is examined directly on either extracted genetic material or nuclei of cells. Similarly, when the polytene chromosomes of *Chironomus* larvae are exposed to copper sulphate, it inhibits the function of Balbiani rings. Severe chromosomal disarrays are caused by the effect of cadmium sulphate's on banding and puffing in polytene chromosomes *in vitro*. *Chironomus* larval chromosomes AB, CD, EF contain a expressive nucleolar organizer (NOR) and elaborated Balbiani rings (BR1 and BR2) in chromosome G. Due to their high sensitivity to stress (Diez et al., 1990; Michailova et al., 1996, 1998), NORs and BRs make suitable models for investigating genotoxicity. *Chironomous* larvae have been shown to be highly susceptible to environmental stress (Hopkin, 1989), and therefore have been utilized for numerous toxicological studies (Warwick 1988; Timmermans et al., 1989). The genotoxic effects of lead, chromium, copper, and cadmium on the structural and functional arrangement of the polytene chromosomes in the salivary gland of Chironomus larvae have been investigated in this study. We have also compared the survival times of larvae after exposure to the above heavy metals.

Materials and methods

The fourth instar larvae of *Chironomus* used in this experiment were obtained from their natural habitat that is a ditch at Department of Botany, University of Allahabad (figure 1).

Figure 1: Fourth instars larva of Chironomus as seen by naked eye.

The larvae were exposed to three different concentrations (10 mg/ml, 5mg/ml and 1mg/ml) of heavy metal salts selected for this study, i.e., ammonium dichromate, lead acetate, cadmium sulphate and copper sulphate (table 1). The exposure of heavy metals was given for 24 hours and 48 hours after which the salivary gland chromosomes were isolated and visualized. A control was also set up in which no chemicals were added.

Acetocarmine stain (2 %**)** was prepared by dissolving 2 g carmine in 100 ml of 45% warm acetic acid. Heating was continued for 15-20 minutes while stirring. The stain was filtered into dark bottles and stored at 4 ºC.

For karyological analysis**,** only fourth instar larvae were used. For preparation of polytene chromosomes, dissection of *Chironomu*s larvae was performed by placing larva on a slide with a drop of water or saline solution for convenient dissection. The head and tail regions were identified under low power of dissecting microscope. The small head is brownish in color with a few simple eyes and movable mouth parts while tail is characterized by the presence of tufy gills. The salivary glands (two flat and whitish structures) were removed from the rest of the body by holding the larva firmly at a short distance behind the head with a pair of extra fine forceps and pulling the head by downward and forward movement of another pair of forceps. The salivary glands were stained by adding 2-3 drops of acetocarmine stain for 10-15 minutes. After that, a cover slip was placed over the gland and the gland was squashed gently under the folds of blotting paper with thumb or blunt end of pencil until the gland was spread out to faint purple monolayer. The slide was examined under the microscope in 10x objective. The area was scanned and focused to a region containing dividing cells showing polytene chromosomes and switched to 40x for clear view.

Hagele (1971) and Kiknaaze et al. (1991) produced standard polytene chromosomal maps of *Chironomus* larvae, which were used to compare the reactions of exposed and control larvae at various concentrations and exposure times. According to Beerman's (1971) scoring system, three levels of puffing (high = "++", intermediate = "+", low or nil = "-") for each of the two homologues were scored in order to determine the activity level of the Balbiani Rings (BRs) and NORs from the control and treatment material. Mortality rate was analysed by exposing larva to different concentrations (10 mg/ml, 5mg/ml,1 mg/ml) of ammonium dichromate, lead acetate, cadmium sulphate and copper sulphate and recording the time of death of larvae at different concentrations of different chemicals.

Results

The diploid chromosome number of polytene chromosome of *Chironomus* is 2n=8. The first (AB), second (CD), and third (EF) chromosomes are metacentric, whereas the fourth (G) is telocentric. Band sequences of all *Chironomus* larvae observed here were similar to those described by Michailova (1987). A dark knob like region characterized as the centromere region was present in the chromosomes. The first second and third chromosomes had one nucleolus localized in the middle. All chromosome arms participated in ectopic pairing. Chromosome G contained two BRs (BR2 and BR1) with high (++) and intermediate (+) levels of activity. In arms C and G, active (either ++ or +) puffs were seen in all of the NORs on chromosomes AB, CD, and EF (figure 2).

Table 1: Effect of heavy metal exposure on puffing activity, Balbiani ring and Nucleolar Organizer region NORs, represented by (++) high, (+) intermediate and (-) low.

Heavy metal salts	**Concentrations**	**Puffing activity**		**Balbiani ring**		**NORs**	
1. Ammonium dichromate		24 h	48 h	24 h	48 h	24 h	48h

	10 mg/ml	+	-	+	+	+	+
	5 mg/ml	-	+	+	+	+	-
	1 mg/ml	-	-	+	+	+	-
2. Lead acetate	10 mg/ml	++	+	++	+	++	+
	5 mg/ml	+	-	+	+	+	-
	1 mg/ml	-	-	+	+	-	+
3. Copper sulphate	10 mg/ml	-	-	+	-	-	+
	5 mg/ml	-	+	+	++	+	-
	1 mg/ml	+	-	++	+	++	+
4. Cadmium sulphate	10 mg/ml	-	-	+	-	+	-
	5 mg/ml	+	-	+	+	+	-
	1 mg/ml	-	-	+	+	-	-

The impact of the metals on the puffing activity, Balbiani ring and NORs has been summarized in table 1 and marked in figures 3-6. According to the results obtained, a reduction of puffing activity was observed after exposure of heavy metals for 48 hours. The puffing activity was also reduced after 24 hours exposure in case of 10 mg/ml cadmium sulphate and copper sulphate, whereas intermediate level of activity was present after 24 hours exposure to 10 mg/ml ammonium dichromate. Interestingly, even after exposure to lead acetate at 10 mg/ml, a high level of puffing activity was observed, implying tolerance to lead acetate at this concentration. However, the activity reduced after 48 hours to an intermediate level. Balbiani ring activity was not inhibited after 24 hours exposure of heavy metals at different concentrations. However, it was inhibited after 48 hours exposure to cadmium sulphate and copper sulphate at 10 mg/ml. NORs were still intact after 24 hours exposure of heavy metals except in the case of copper sulphate. However they were found to be inhibited after 48 hours exposure to cadmium sulphate and lead acetate.

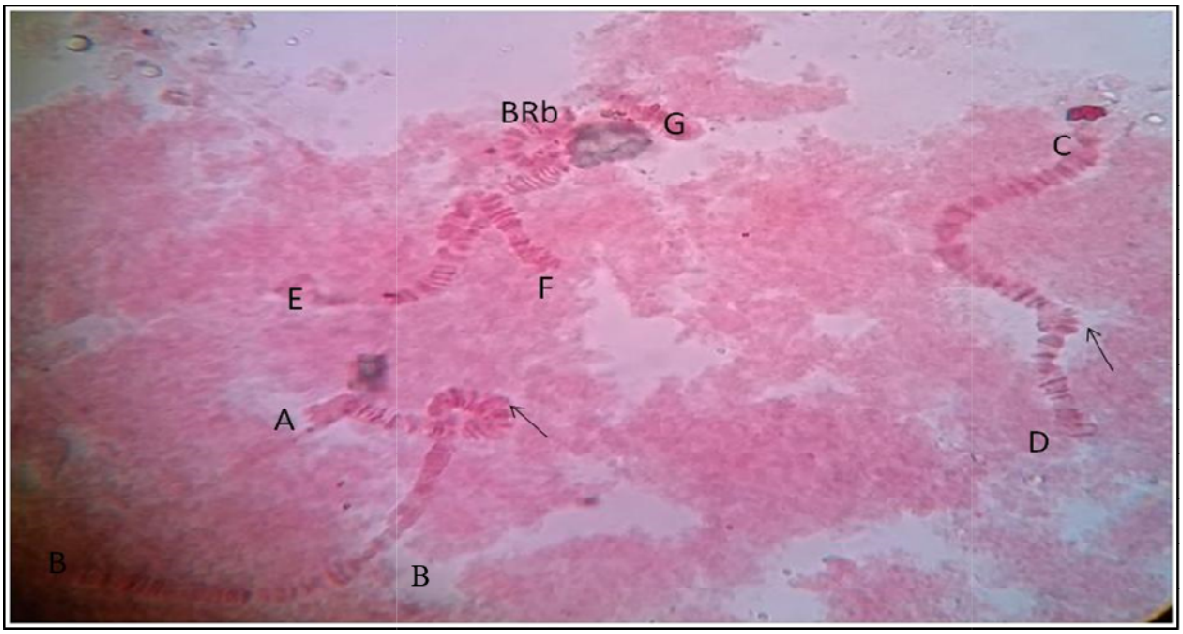

Figure 2: Standard idiogram of the polytene chromosomes of Chironomus larvae. The four chromosomes namely AB, CD, EF, G and Balbiani ring (BRb) have been marked. Puffs have been marked by arrows.

Effect of ammonium dichromate treatment on *Chironomous* larvae resulted in a rise in functional defects in the polytene chromosomes of the treated larvae (figure 3) relative to the control group. Decondensed centromeres were present in all of the polytene chromosomes, but they were more prevalent in chromosomes CD and EF. The granular structure of telomeres was frequently seen. Ectopic connections were more frequently observed between telomeres of chromosomes than between intercalary regions in treated larvae compared to controls. Polytene chromosome with a grain structure was visible in the treated larvae in a mosaic state. The overall frequency of aberration for the various concentration treatments did not change significantly, therefore we also compared with control and with 24h and 48h time period exposure. As compared to 24 h exposed larvae, 48 h exposed larvae show more chromosomal aberrations. Chromosome G was shown to have both BRs deleted in certain treated larval cells, resulting in the appearance of pompons, as reported by Michailova et al (1996). Located in the salivary glands primary lobe of the salivary gland, these pompons had an extremely streamlined appearance.

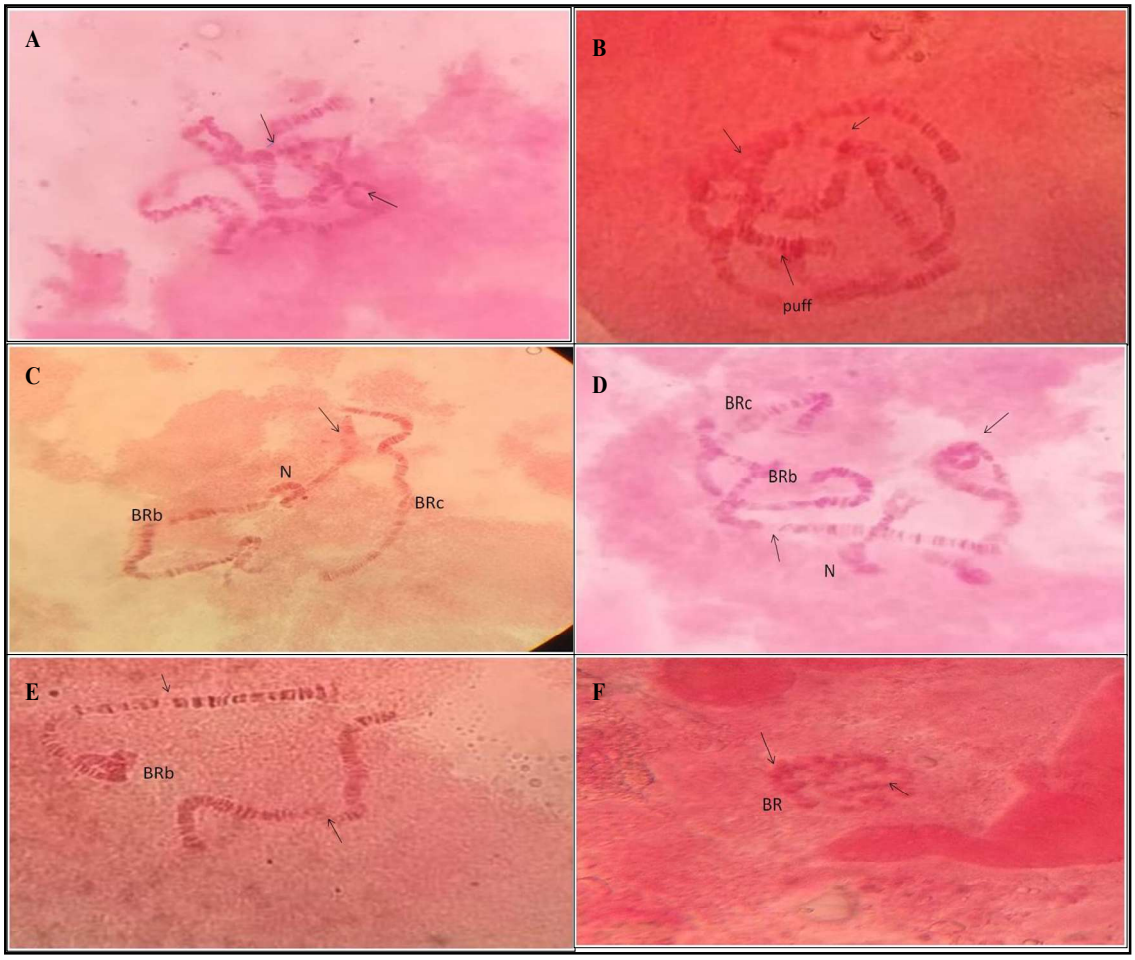

Figure 3: (A), (B) 10 mg/ml, (C), (D) 5 mg/ml and (E), (F) 1mg/ml. An exposure for 24 hours was given in (A), (C) and (E) and for 48 hours in (B), (D) and (F). Arrows indicate deconstruction of telomere arm. BR, BRb, and BRc: Balbiani rings. Visible genotoxic effects of ammonium dichromate were chromosome decondensation, deletion of Balbiani rings grain like structure in chromosomes.

Impact of lead acetate exposure on polytene chromosomal functional activity (figure 4) included decondensed centromeres occurring at very low frequency (after all exposure concentrations) after treatment with lead, as compared to control. Greater degree of decondensation at high concentration and 48 hours exposure was observed as compared to intermediate concentration and 24 hours exposure. The appearance of the Balbiani rings BR2 and BR1 varied according to the absolute and relative level of puffing inflation. BR2 exhibited a much higher functional activity than did BR1 in control and low (1mg/ml), intermediate (5mg/ml) and high (10mg/ml) concentration of lead acetate due to fall in BR2 activity with increased time period of exposure. The number of chromosomes showing high activity of both BR1 and BR2 decreased after exposure of different concentrations of lead acetate. The level of high activity of NORs in salivary gland chromosomes decreased, following exposure to 10 mg/ml, 5mg/ml, and 1mg/ml lead acetate for 24 h to 48 h as compared to control. The activity of the puff in arm G of both lead acetate exposed and control chromosome was very low and did not change with exposure.

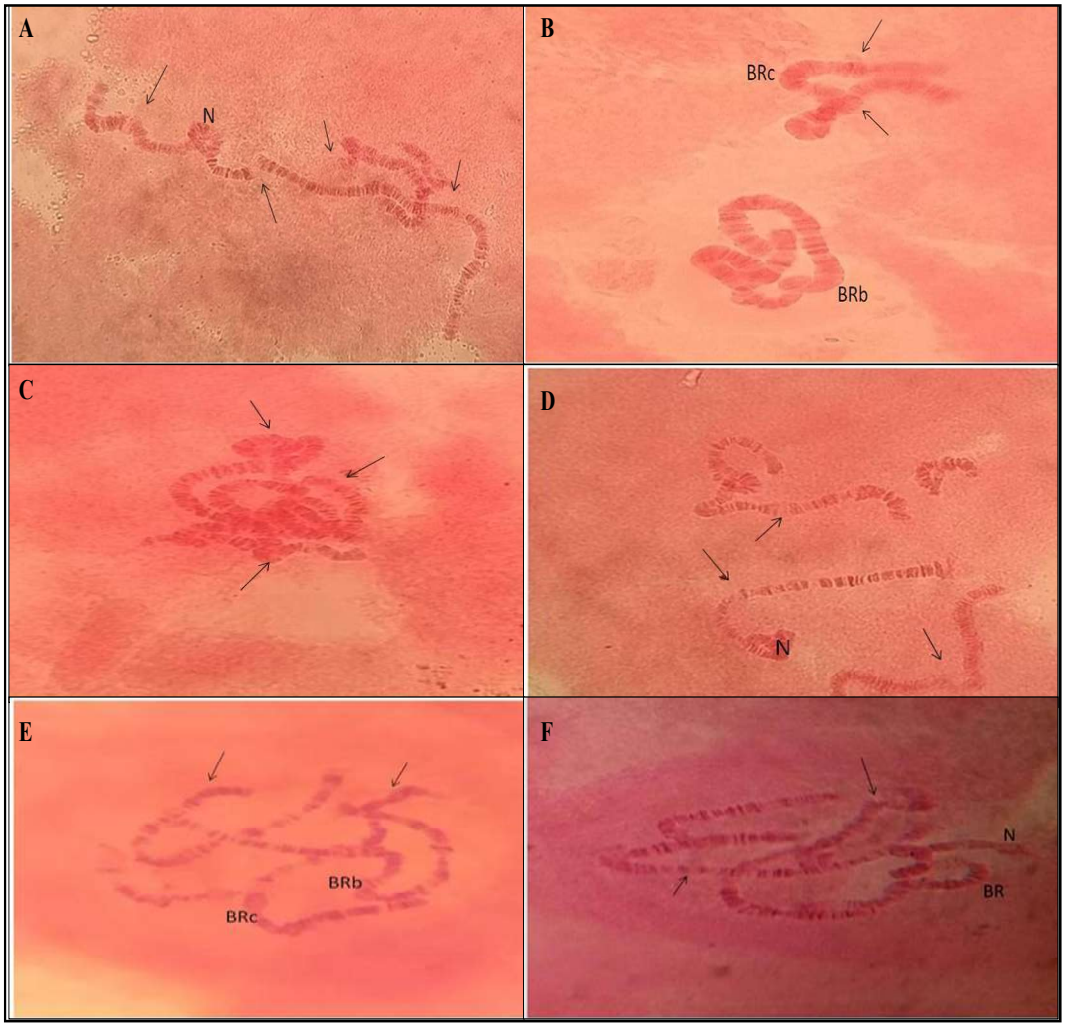

***Figure 4:** (A), (B) 10 mg/ml, (C), (D) 5 mg/ml, (E), (F) 1 mg/ml. An exposure for 24 hours was given in (A), (C) and (E) and for 48 hours in (B), (D) and (F). Visible genotoxic effects of lead acetate were decondendation of chromosomes, constriction of chromosome with intermediate (+) puffing, decreased puffing activity and decreased Balbiani ring structure.*

The activity of Balbiani rings (BR1 and BR2) and the nucleolar organizing region (NOR) in chromosomes was observed to be significantly impacted by copper sulphate (figure 5) without appreciable variations, BR2 and NOR were decreased by the same concentration of copper sulphate.

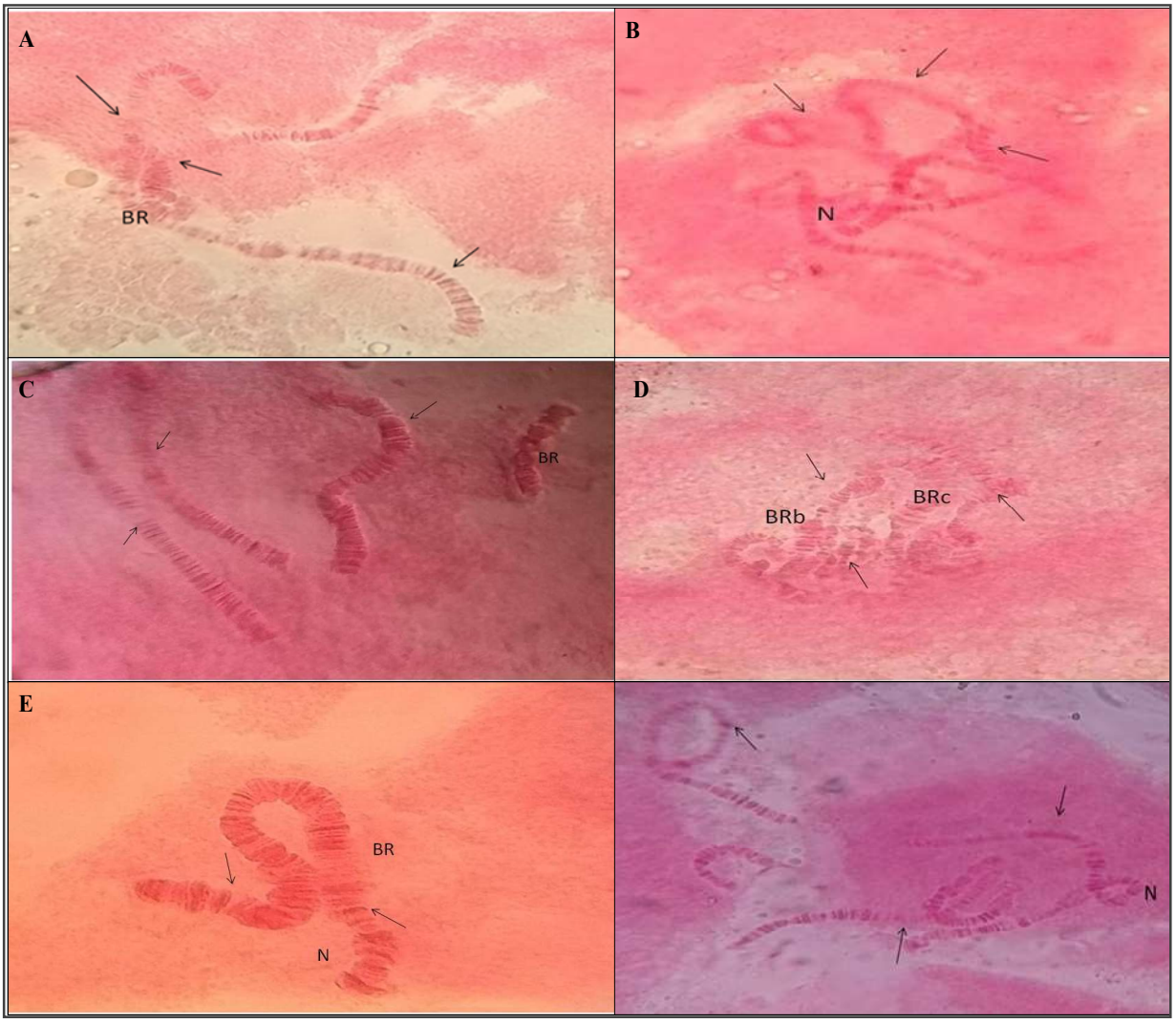

Figure 5: (A), (B) 10 mg/ml, (C), (D) 5 mg/ml, (E), (F) 1 mg/ml. An exposure for 24 hours was given in (A), (C) and (E) and for 48 hours in (B), (D) and (F). Observable genotoxic effects of copper sulphate were decondensation, constriction of chromosomes, decreased puffing activity and Balbiani rings.

The effect of different concentrations and different time periods of exposure of cadmium sulphate was observed (figure 6). It causes alteration in the banding pattern and puffing of the polytene chromosomes in the saliva of larvae was the result. Gene malfunction was clearly visible because puff induction and regression were found in the puffing study.

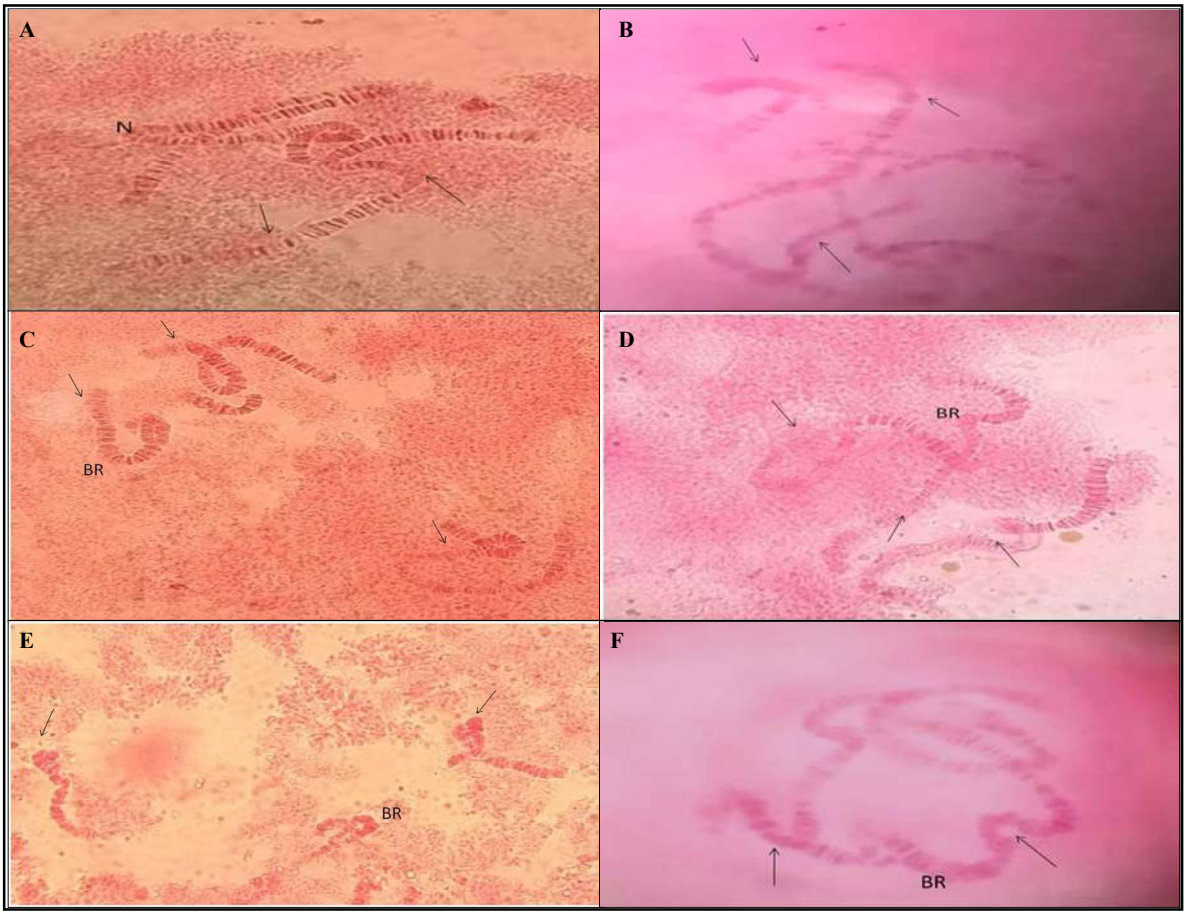

Figure 6: (A), (B) 10 mg/ml, (C), (D) 5 mg/ml, (E), (F) 1 mg/ml. An exposure for 24 hours was given in (A), (C) and (E) and for 48 hours in (B), (D) and (F). Observable genotoxic effects of cadmium sulphate were low (-) puffing activity and decondensation of chromosomes and damaged or decreased Balbiani ring.

Mortality analysis of *Chironomus* larvae after exposure to different concentrations (10 mg/ml, 5mg/ml and 1 mg/ml) of ammonium dichromate, lead acetate, copper sulphate and cadmium sulphate showed that the survival time varies from different concentrations of different chemicals (figure 7). The survival time of larvae after addition of 10 mg/ml of the heavy metals was smallest in case of copper sulphate (8.5 hours) and longest in case of lead acetate (16 hours). After addition of 5 mg/ml of the heavy metals, larvae survived for the shortest period in lead acetate (7 hours) and for the longest duration in ammonium dichromate (16.5 hours). 1 mg/ml of heavy metals shortened the survival time of larvae in cadmium sulphate the most (4 hours), whereas in copper sulphate, the larvae survived for 13 hours. Thus, although the survival times of larvae on exposure to different concentrations of heavy metals did not seem to be dependent on concentration, but definitely it shortened the life span of the larvae.

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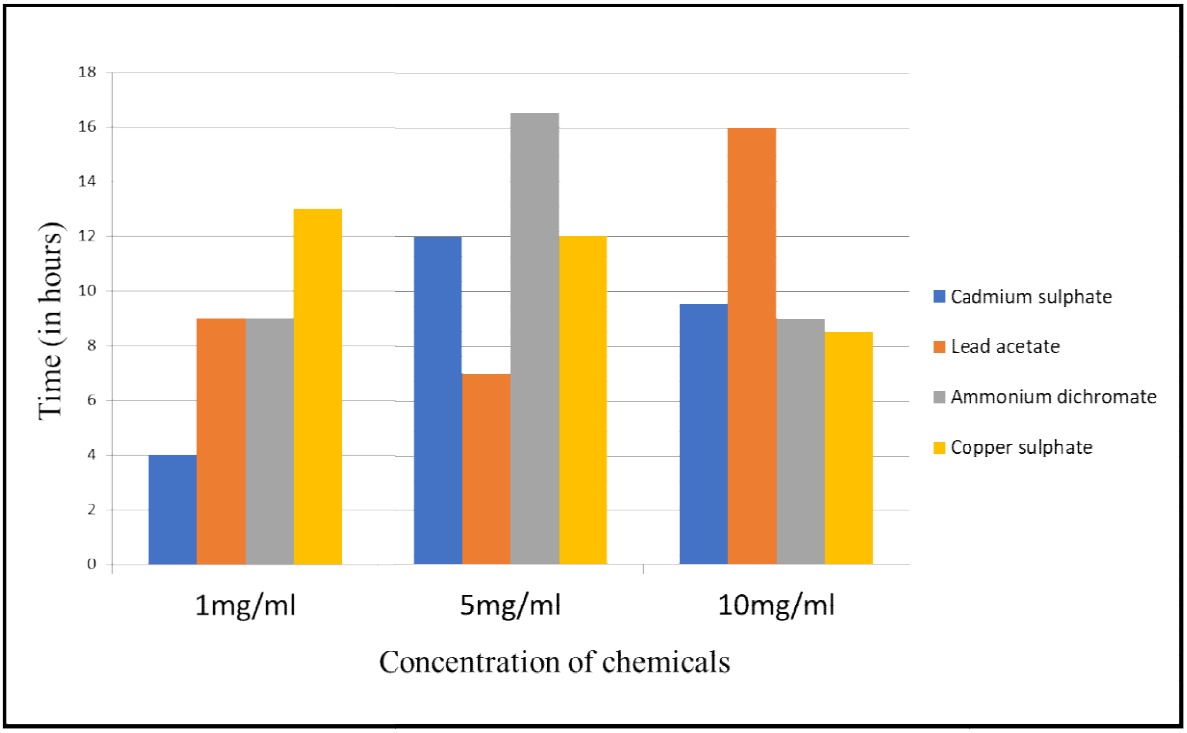

Figure 7: Comparison of survival times of Chironomus larvae after addition of different concentrations (1 mg/ml, 5 mg/ml and 10 mg/ml) of ammonium dichromate, lead acetate, copper sulfate and cadmium sulfate

Discussion

Chironomidae has been used as a bioindicator in several researches. These studies indicate a connection between larval mortality and the immediate toxic effects of several kinds of polluting substances (Hudson & Iborowsky, 1996). Our results exhibit a association between acute exposure to different doses of ammonium dichromate, lead acetate, copper sulphate and cadmium sulphate and their genotoxic effects on the chromosomes, as compared to control (eg. decondensation of the telomeres of chromosomes C, A, and G, creation of a pompon-like chromosome G, deletion of BRb and BRc, and a noticeably increased incidence of somatic amplifications of three areas of chromosome EF).

Ammonium dichromate treatment induced changes in BRs and puffing that are similar to those observed in larvae of *Chironomus* from heavy metal polluted areas near Turin (Michailova et al., 1998). Similar changes in the puffing activity of Balbiani rings (BRs) have been reported in literature. Ammonium dichromate treatment caused BRs and puffing alterations that resembled those seen in *Chironomus* larvae from heavy metal contaminated sites close to Turin (Michailova et al., 1998). As reported by the literature, a similar alteration occurs in the Balbiani rings' (BRs') puffing behavior. Balbiani rings regression occurs by heat shocks (Barttino et al., 1998), galactose (Diez et al., 1990), and sugar treatments (Beermann, 1973). Therefore, it is possible to hypothesize that the BR system reacts to different stressful circumstances. Owing of the folding back of the region immediately after BRc, we saw that in some of the treated larval chromosomes G, the nucleolus appeared to be at the end of the chromosomes. *Chironomus*

larvae residing at a heavy metal-polluted environment close to Turin have shown this phenomenon on many chromosomal G (Michailova et al., 1998). This folded-back look of the apical portion of the chromosome, along with the creation of pompon-like chromosomes G, can be interpreted as a particular cytogenetic response to stressful environments. Lead acetate exposure also modifies how the polytene chromosomes function suggesting a common reaction to lead stress. Chromosomal puffing of the chromosome arm C was stimulated by lead in dose dependent manner. Lead acetate exhibited high sensitivity in the Balbiani ring as well as the nucleolar organizer of the salivary gland chromosomes in *Chironomus* larvae. It reduced NOR activity in *Chironomus* in this study. Similar results have been observed by Aziz et al. 1(991), suggesting that the synthesis of heat shock protein (HSP) suppresses the NOR activity. Horgen and Griffin (1971) explained the suppression of NOR activity by the inhibition of RNA polymerase I. A similar effect was observed on the BRs in *Chironomus* after treatment with copper sulphate and cadmium sulphate. Specific reactions were observed in BRb and BRc showing slight activity or collapse, while BRb was activated. Larvae exposed to galactose have shown similar changes in BRs activity (Diez et al., 1990). Similarly, a shift in the relative size of BR1 and BR2 in *Chironomus* has been observed after ethanol treatment (Yagi, 1984). The results of this study demonstrated that the both NORs and BRs have transcription mechanisms that respond to a range of stressors similarly, and these structures are very sensitive to many stressors.

In these experiments, treatment with heavy metals enhanced the degree of compactness of centromeric heterochromatin, and this may be related to the creation of proteins that contribute to chromatin condensation. These findings are consistent with Morales (2016) hypothesis that the metal indirectly affects genetic processes. The data generated here demonstrates that, while not always in proportion to exposure concentration, a metal can cause a variety of functional changes in *Chironomus* polytene chromosomes. The results of this study confirm the genotoxicity of heavy metals, and also the potential for enhanced activity of the Chironomidae genome in the environmental stress. These responses could be used as cost effective and receptive biomarkers for detecting a range of genotoxic agents beneath natural environment. Mortality analysis after exposure to heavy metals showed that the survival time period of larvae after exposure is ~ 9 hours on an average. Mortality analysis is also useful as environmental bioindicator.

Conclusion

After study of impact of heavy metals on polytene chromosomes of *Chironomus* larvae, it can be concluded that although metals are important for some metabolic activities, but if their concentrations increase more than normal for long periods of time, they show genotoxic effects on *Chironomus* larvae. Heavy metals not only affect the life-span of the larvae, but also alter the structural and functional characteristics of polytene chromosomes. Long term exposure causes death of the larvae. Therefore, *Chironomus* larvae have great potential to be used as bioindicator of heavy metal stress.

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