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RESEARCH ARTICLE

A COMPARATIVE STUDY OF THE EFFECT OF TWO DIFFERENT DOSES OF GAMMA RAYS ON MITOTIC CHROMOSOMES OF MOUSE *MUS MUSCULUS*

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SUMMARY The living world is exposed to hazardous radiations from time to time as for example X-rays from X-ray machine, gamma rays from Co^{60} for cancer therapy and even uv rays from the sun. Ionizing radiations such as X-rays and gamma rays have been reported to create free radicals within a cell and thus cause damage to chromosomes in the living cells and tissues. To evaluate the effect of such ionizing radiation, the effect of 2 different doses of gamma rays on common mouse *Mus musculus* has been studied here. Both sexes of mouse were exposed to whole body irradiation of gamma rays in 2 different sets of 0.8 Gy and 2.4 Gy. Various types of chromosomal aberrations were evaluated from bone marrow cells of the mouse, after 5 different time intervals viz., 16 h, 48 h, 1 wk, 2 wks and 4 wks. It had been observed that in case of both the doses of irradiation, the higher percentage of aberrations have been observed after 48 h of exposure (11.66% in case of 0.8 Gy and 19.01% in case of 2.4 Gy) while there is a decline in aberration after 4 wks of exposure (3.48% in case of 0.8 Gy and 6.66% in case of 2.4 Gy). On the other hand, a total of 9 different types of chromosomal aberrations were detected. The highest frequency of aberration was observed with rabbit ear chromosome for both the doses (1.85% in case of 0.8 Gy and 2.59% in case of 2.4 Gy), while ring chromosome was scored at the minimum level (0.22% and 0.20% for 0.8 Gy and 2.40 Gy respectively). Statistical analysis of the data reveals that the aberrations are non-random in distribution; they are somewhat time-dependent and centromeric regions of some chromosomes are most vulnerable to γ irradiation. Moreover, 2.4 Gy dose has induced sub-lethal physiological changes in the mouse.

Keywords: Gamma rays, chromosomal aberrations, *Mus musculus*, tumour, physiological change.

INTRODUCTION

Fluctuating environmental conditions and other natural parameters are enough to induce a great variety of mutations on natural biota. From time immemoreal, the living world is exposed to hazardous ionizing radiations viz., X-rays, gamma rays (γ rays) and nonionizing radiations such as uv rays. The effects of various physical mutagens as ionizing radiations are known to cause severe damage to living tissues, including

their clastogenic effect on chromosomes within living cells. The pioneering discovery by Muller (1927) on artificial mutagenesis in *Drosophila* opened a new horizon to the cytogenetic study of chromosomal aberrations. Consequently, several workers had done extensive work on the exposure of these radiations and genomic instability in wide range of animal and plant models. Some of the distinguished works in this field are of Stadler (1928), Goodspeed & Olson (1928), Carlson (1938, 1940, 1941a, 1941b), Carlson et al. (1949) and Manna & Mazumder (1962, 1967, 1968). Investigations with γ rays were carried out extensively throughout the globe by various researchers. The effect of γ rays from Co^{60} source on a triturated thymidine injected L-P59 mouse fibroblasts and ascites tumour cells both in vivo and in vitro had been studied by Dewey & Humphery (1962). When mouse zygotes are exposed to X-rays, they lead to chromosomal aberrations in foetal skin fibroblast cells, which had been reported for the first time by Pampfer & Streffer (1989).

Not only physical mutagens, but also studies with chemical mutagens have been widely reported. Exposures to chemical mutagens such as bleomycin (Taylor et al. 1979) 4-nitroquinolonel-1-oxide (Smith & Paterson 1980) and neocarzinostatin (Shiloh et al. 1982) have been found to be genotoxic too. Nobuyuki et al. (2001) reported the intense toxic effect of 1- and 3-nitroazabenzo[a]pyrene and their N-oxides were isolated from diesel particulates in mice. Madrigal-Bujaidar et al. (2010) reported that 2 drugs, imipramine and desipramine used for the treatment of depression, are also found to cause chromosomal aberrations in mouse bone marrow cells. Recently, D'Auria Vieira de Godoy et al. (2021) investigated the effect of high dose rate and chronic low dose rate of gamma irradiation on BL6 mice, and found that high dose of the radiation is more lethal by forming more micronuclei in bone marrow cells of the animals.

We have extensively studied the effect of physical mutagens on a wide range of animal models and also discovered for the first time anti-neoplastic and anti-tumour activity of phyto-active constituents in leaf extract of *Barleria lupulina* (Das &Sur 2012, Sur & Das 2012, Das 2013). The present study deals with radiation induced chromosomal damage by using 2 different doses of γ-rays, 0.8 Gy and 2.40 Gy on mitotic chromosomes of mouse *Mus musculus*.

MATERIAL AND METHODS

Adult healthy Swiss albino mouse (*M. musculus*) of both sexes were purchased from the local supplier and maintained with food and water to acclimatize to laboratory conditions. After acclimatization, the animals were subjected to whole body γ irradiation of 2 different doses, 0.8 Gy and 2.40 Gy. $_{27}Co^{60}$ was used as the γ ray source. The mice were caged in special container and subjected to γ rays in 2 different sets for 2 different doses. A total of 10 mice (5 males and 5 females) were used in the treatment for each time interval. Therefore, over all 50 mice were used for 0.8 Gy dose at different time intervals (16h, 48h,

1wk, 2wks, 4wks) and similarly 50 mice were used for 2.40 Gy dose at the same time intervals. 2 female mice and 3 male mice died after 4 wks post exposure with 0.8 Gy and 2.40 Gy doses respectively.

After exposure to radiation, the mice were transferred to laboratory. They were chloroformed and sacrificed after 5 different time intervals i.e. 16 h, 48 h, 1 wk, 2 wks and 4 wks. Mitotic chromosomes of the mice were studied from metaphase plates prepared from femur bone marrow cells. The femur bones of both hind legs were dissected out and bone marrow was suspended in 1% sodium citrate solution and a uniform suspension was obtained by flushing. Fixation was done in acetic-alcohol (1:3 v/v). Slides were prepared by dropping the cells onto chilled slides maintained at -5° C, and staining was done by Giemsa staining solution for one and a half to 2 h. Scoring of data was done by using 900 cells for each time interval. Standard statistical tools were employed to analyze the data.

OBSERVATIONS

A total of 900 mitotic cells for each hour, summing up to 4500 cells in 5 different time-intervals were studied in untreated mice which served as control for 0.8 Gy dose. The chromosome number is 2n = 40. Therefore, all total 180000 mitotic chromosomes were studied in this whole control series. The maximum chromosomal aberrations were observed with sub-chromatid gap and centromeric dissociation (0.07% for both), while minimum aberrations were obtained with ring chromosome (0.01%). Therefore, no significant aberrations were found in the control series. Similarly for 2.4 Gy dose, 4500 bone marrow cells summing up to 180000 mitotic chromosomes were studied. Negligible chromosomal aberrations have been observed here also, the highest being 0.2% with centromeric dissociation and lowest being 0.03% with ring chromosome (Table 1).

In treated series, a total of 4500 cells and 180000 chromosomes were studied. Here, at 0.8 Gy γ ray exposure, 9 different types of chromosomal aberrations were observed, such as chromatid break, sub-chromatid gap, iso-chromatid gap, centromeric dissociation, iso-chromatid break, rabbit-ear chromosome, rabbit-ear chromosome with gap, translocation and ring chromosome, (Table 1, Figs 5–8). Of these, highest frequency of aberrations were obtained with rabbit-ear chromosome (1.85%) while the least was obtained with ring chromosome (0.22%) (Table 2, Fig. 1). The other types of aberrations lie within these 2 values. On comparison with time, the highest aberrations were observed at 48 h (11.66%) and lowest at 4 wks (3.48%). Therefore, it was pragmatic that chromosomal aberrations increased slowly from 16 h (10.57%) to 48 h (11.66%) post exposure, and then decreases (3.48%) and becomes negligible after 4 wks of exposure (Table 1, Fig. 2). Therefore, a total of 7.96% of chromosomal aberrations had been scored in mice treated with 0.8 Gy γ rays (Table 1).

TABLE 1: 0.8 Gy γ ray irradiated chromosomal aberrations in mice.

Duration		No. of cells	No. of chromo-somes	Types of aberrations: Chromatid break	Sab-chromatid gap	Iso-Chromatid gap	Centromeric dissociation	Iso-Chromatid break	Rabbit-ear chromosome	Rabbit-ear chromo-some with gap	Trans-location	Ring chromo-some	Total aberrations	Aberrations (%)
16 h	C	900	36000	0	54	0	12	9	6	24	6	6	117	0.33
	T	900	36000	600	699	399	306	615	498	198	414	75	3804	10.57
48 h	C	900	36000	6	33	9	9	12	18	15	6	6	114	0.32
	T	900	36000	1236	675	225	111	129	786	453	468	114	4197	11.66
1 wk	C	900	36000	15	0	3	54	6	45	24	3	0	150	0.42
	T	900	36000	675	225	138	114	45	1578	198	84	45	3102	8.62
2 wks	C	900	36000	33	0	3	36	15	15	6	9	6	123	0.34
	T	900	36000	345	414	144	54	42	414	69	345	138	1965	5.46
4 wks	C	900	36000	6	36	18	12	0	12	3	6	0	93	0.26
	T	900	36000	189	243	75	381	72	45	63	165	18	1251	3.48
Total aberrations	C	4,500	180000	60	123	33	123	42	96	72	30	18	597	0.33
	T	4,500	180000	3045	2256	981	966	903	3321	981	1476	390	14319	7.96
Total % of aberrations			C	0.03	0.07	0.02	0.07	0.02	0.05	0.04	0.02	0.01		
			T	1.69	1.25	0.55	0.54	0.50	1.85	0.55	0.82	0.22		

C, Control; T, Treated.

TABLE 2: 2.40 Gy γ ray irradiated chromosomal aberrations in mice.

Duration		No. of cells	No. of Chromo-somes	Types of aberrations: Chromatid break	Sub-chromatid gap	Iso-chromatid gap	Centromeric dissociation	Iso-chromatid break	Rabbit-ear chromo-some	Rabbit-ear chromo-some with gap	Trans-location	Ring chromo-some	Total aberrations	Aberrations (%)
16 h	C	900	36000	54	9	45	75	27	48	15	36	6	315	0.88
	T	900	36000	906	1128	915	1350	228	669	234	456	24	5910	16.42
48 h	C	900	36000	39	48	36	114	18	45	12	9	3	324	0.90
	T	900	36000	1668	834	486	1644	87	1086	723	261	54	6843	19.01
1 wk	C	900	36000	48	24	15	75	51	57	54	33	15	372	1.03
	T	900	36000	714	474	294	768	75	1245	354	78	75	4077	11.33
2 wk	C	900	36000	78	39	18	15	12	48	24	21	6	261	0.73
	T	900	36000	699	306	315	201	99	912	615	102	45	3294	9.15
4 wk	C	900	36000	45	54	12	84	57	96	51	78	27	504	1.4
	T	900	36000	225	258	72	309	162	753	303	159	156	2397	6.66
Total aberrations	C	4,500	180,000	264	174	126	363	165	294	156	177	57	1776	0.99
	T	4,500	180,000	4212	3000	2082	4272	651	4665	2229	1056	354	22521	12.51
Total % of aberrations			C	0.15	0.10	0.07	0.20	0.09	0.16	0.09	0.10	0.03		
			T	2.34	1.67	1.16	2.37	0.36	2.59	1.24	0.59	0.20		

C, Control; T, Treated.

TABLE 3: Statistical analysis of the effect of 0.8 Gy γ rays on mitotic chromosomes in *M. musculus*.

Aberration statistics	Chromatid break	Sub - chromatid gap	Iso-chromatid gap	Centromeric dissociation	Iso-chromatid break	Rabbit-ear chromo-some	Rabbit-ear chromo-some with gap	Trans-location	Ring chromo-some	Pooled
Control ± SE	1.76	3.25	0.45	2.68	0.79	2.05	1.34	0.29	0.45	3.50
Treated ± SE	54.59	30.99	17.05	19.29	33.39	78.28	21.56	22.36	6.66	168.4
Control CD at 5%	3.44	6.36	0.88	5.26	1.54	4.02	2.62	0.57	0.88	6.87
Treated CD at 5%	106.78	60.61	33.34	37.74	65.30	153.11	42.06	43.74	13.04	329.43
Control CD at 1%	4.52	8.36	1.15	6.91	2.02	5.29	3.44	0.74	1.15	9.02
Treated CD at 1%	140.57	79.79	43.89	49.68	85.97	201.56	55.37	57.58	17.16	433.69
t values	4.58	5.21*	4.66	3.87	3.73	5.32*	5.41	6.64**	7.65**	8.65**
$\chi 2$ values	51.09**	24.07**	6.12*	72.92**	25.26**	12.13*	7.93*	2.21	3.10	23.05**
r values	-0.31	0.61*	-0.26	-0.57	0.07	0.90**	0.45	0.56*	0.87**	0.48*

Overall F value = 5.28 **; DMR value = 9.98 **; * Significant at 5% level; ** Significant at 1% level.

SE, Standard error; CD, Critical difference; t values, Students t test; χ^2, chi-square; r-value, correlation coefficient; F Value, Analysis of variance; DMR value, Duncan multiple range.

TABLE 4: Statistical analysis of the effect of 2.4 Gy γ rays on mitotic chromosomes in *M. musculus*.

Aberration statistics	Chromatid break	Sub-chromatid gap	Iso-chromatid gap	Centromeric dissociation	Iso-chromatid break	Rabbit-ear chromo-some	Rabbit-ear chromo-some with gap	Trans-location	Ring chromo-some	Pooled
Control ± SE	2.05	2.49	1.97	4.89	2.72	2.90	2.72	3.55	1.34	12.53
Treated ± SE	71.49	50.59	42.89	86.09	8.73	32.15	28.80	20.25	6.94	250.31
Control CD at 5%	4.02	4.89	3.86	9.59	5.34	5.67	5.33	6.96	2.62	24.55
Treated CD at 5%	139.83	98.95	83.89	168.40	17.08	62.89	56.33	40.98	13.58	489.61
Control CD at 1%	5.29	6.42	5.07	12.60	7.01	7.46	7.00	9.15	3.44	32.25
Treated CD at 1%	184.08	130.27	110.44	221.69	22.48	82.79	74.16	53.95	17.88	644.55
t values	4.61*	4.59*	4.29*	4.27*	6.14**	7.97**	6.78**	5.20**	7.19**	8.60**
$\chi 2$ values	43.66**	50.54**	3.29	41.43**	20.32**	19.26**	32.88**	48.30**	3.13	188.94**
r values	-0.30	-0.62**	0.94**	0.72**	0.09	-0.31	-0.48*	-0.09	0.94**	-0.50*

Overall F value = 10.69 **; DMR value = 19.89 **; * Significant at 5% level; ** Significant at 1% level.

SE, Standard error; CD, Critical difference; t values, Students t test; χ^2, chi-square; r-value, correlation coefficient; F Value, Analysis of variance; DMR value, Duncan multiple range.

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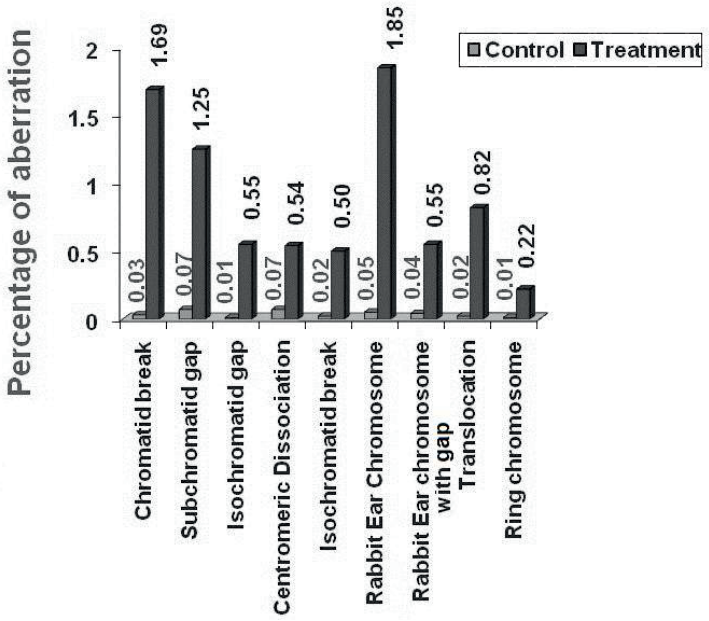

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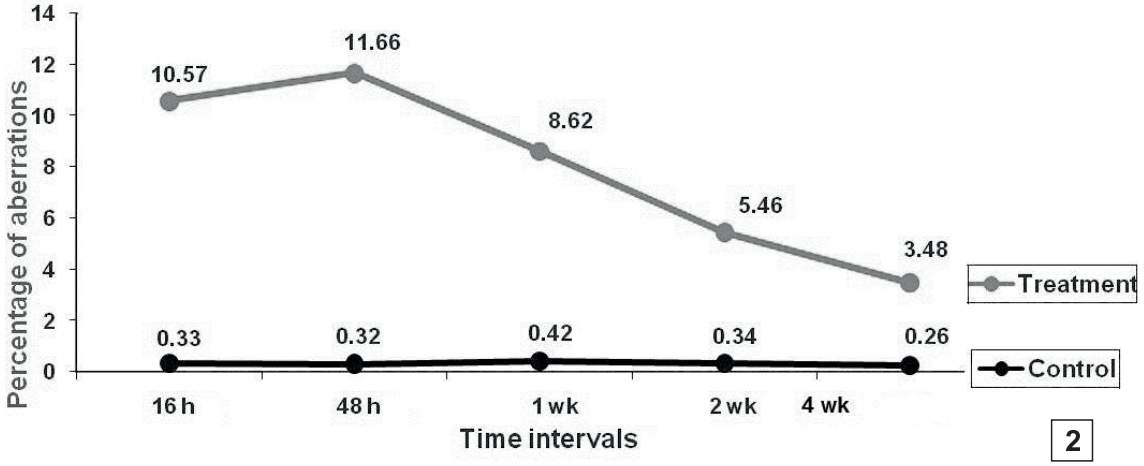

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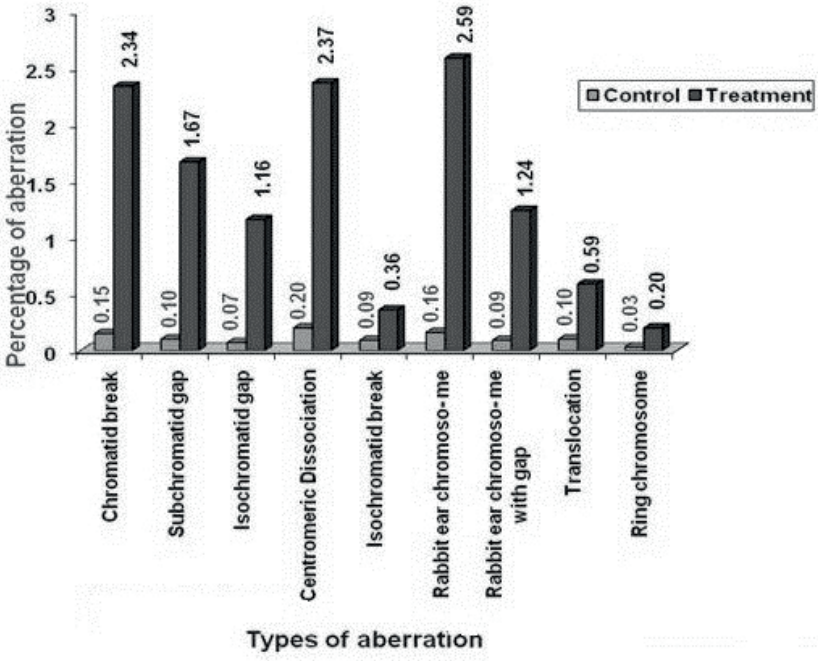
Figs 1 & 2: 1. Bar-diagram showing nine different types of chromosomal aberrations in mice treated with 0.8 Gy γ rays. 2. Line-diagram showing chromosomal aberrations with respect to time in bone marrow cells of mice induced by γ ray (0.8 Gy) and compared with control.



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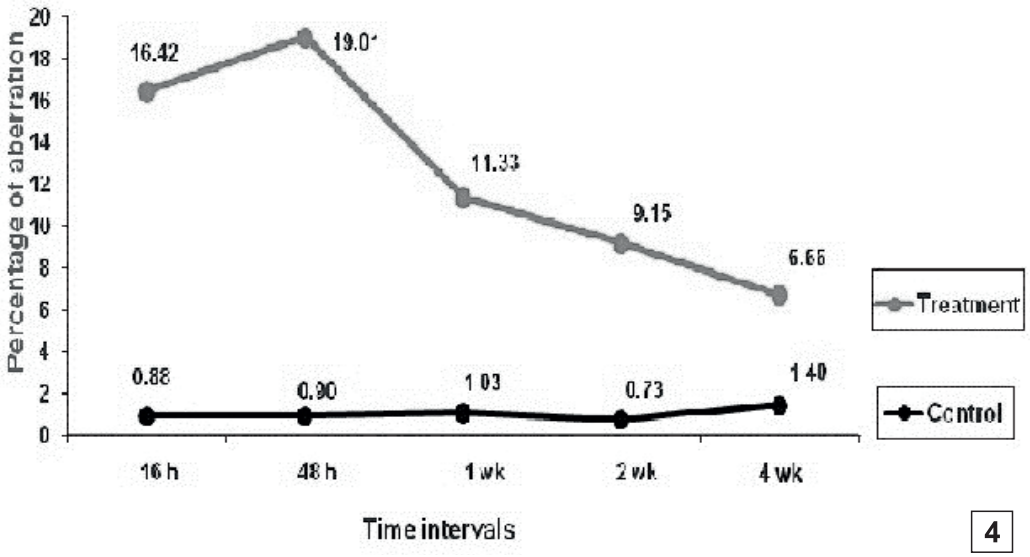

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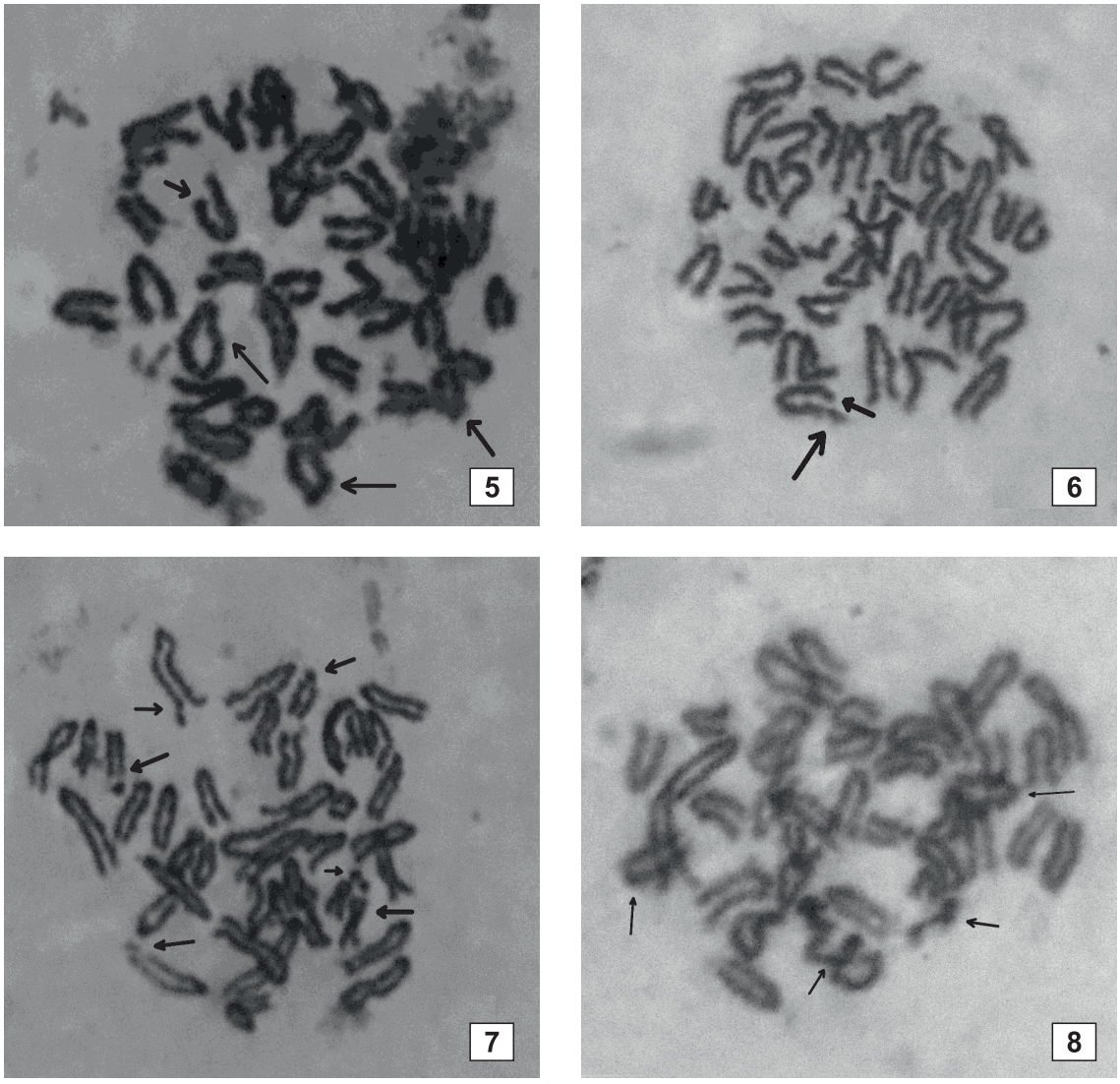
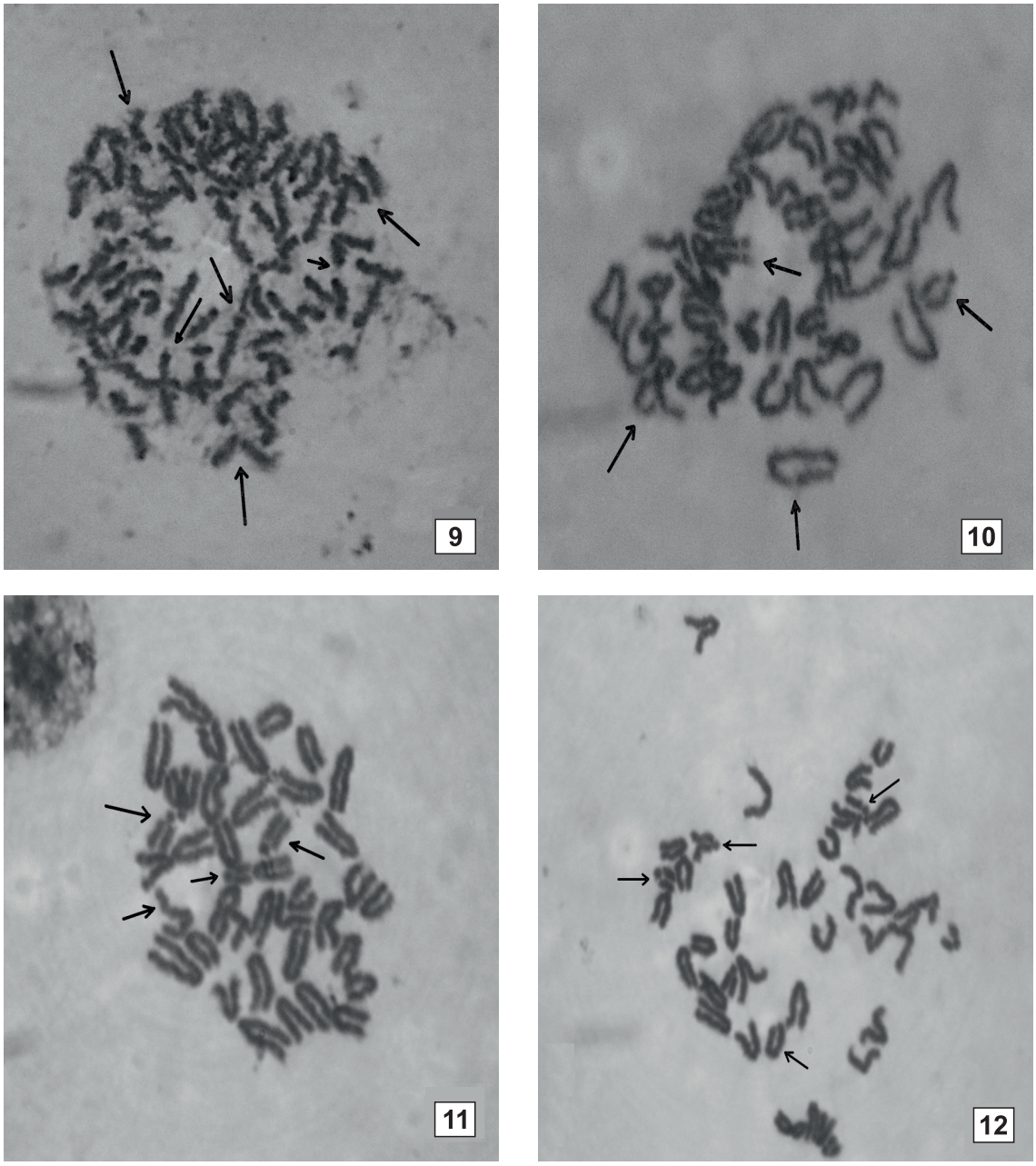
Figs 3 & 4: 3. Bar-diagram showing nine different types of chromosomal aberrations in mice treated with 2.4 Gy γ rays.
4. Line-diagram showing chromosomal aberrations with respect to time in bone marrow cells of mice induced by γ ray (2.4 Gy) and compared with control.

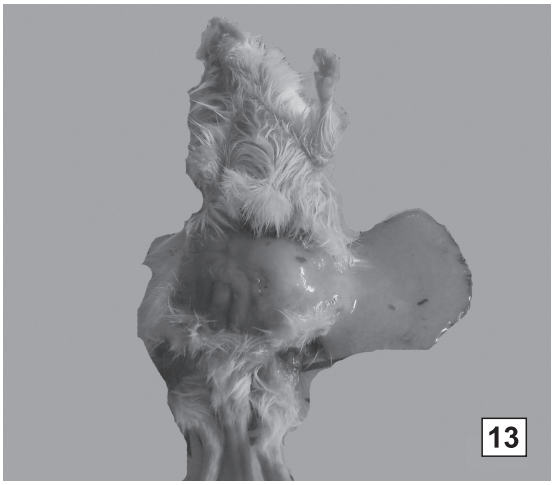


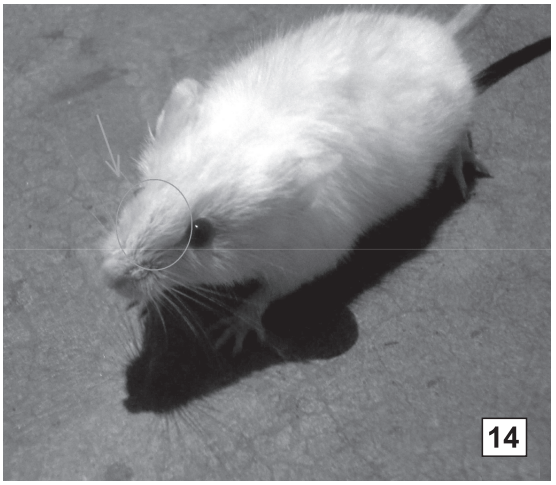
Figs 5–8: Photomicrographs showing various chromosomal aberrations induced by 0.8 Gy gamma rays. 5. Chromatid break, ring chromosomes (arrows). 6. Chromatid break, sub-chromatid gap (arrows). 7. Iso-chromatid gap, rabbit-ear chromosome with gap, rabbit-ear chromosome, chromatid break (arrows). 8. Translocation (arrows).

Statistical analysis of the data reveals that the t-value is significant at 1% level for translocation (6.64) and ring chromosome (7.65), whereas at 5% level it is significant for subchromatid gap (5.21) and rabbit-ear chromosome (5.32). Additionally, the χ^2 value for the treated series are highly significant at 1% level viz., 51.09 for chromatid break, 24.07 for subchromatid gap, 72.92 for centromeric dissociation, 25.26 for iso-chromatid break and over all the χ^2 value for the



Figs 9–12: Photomicrographs showing various chromosomal aberrations induced by 2.4 Gy gamma rays. 9. Rabbit-ear chromosome, chromatid break, rabbit-ear chromosome with gap, centromeric dissociation (arrows). Also physiological aberration of each chromosome is prominent. 10. Iso-chromatid break, sub-chromatid gap. 11. Rabbit ear-chromosome, translocation (arrows). 12. Rabbit-ear chromosome, ring chromosome (arrows).





Figs 13 & 14: Physiological changes. 13. Accumulation of excess peritoneal fluid in stomach of mouse after 16 h of treatment with 2.4 Gy γ rays. 14. Mouse showing tumour (1 cm/0.5 cm) between two eyes above the snout region after 4 wks of treatment with 2.4 Gy γ rays.

pooled data is 23.05. The overall F-value is 5.28 and DMR value is 9.98 which are significant at 1% level i.e., highly significant (Table 3).

In mice treated with 2.4 Gy γ rays 9 different types of structural chromosomal aberrations were observed (Table 2, Figs 9–12). The predominant structural aberrations observed were rabbit-ear chromosomes (2.59%) and lesser frequency the ring chromosomes (0.20%) (Table 2, Fig. 3). In terms of time intervals, the highest frequency of aberrations was observed after 48 h (19.01%) of exposure to γ rays and lowest was after 1 month (6.66%) post exposure (Table 2, Fig. 4).

The t-value is significant at 1% level for isochromatid break (6.14), rabbit-ear chromosome (7.97), rabbit-ear chromosome with gap (6.78), translocation (5.2) and ring chromosome (7.19), whereas the t-value is significant at 5% level for chromatid break (4.61), subchromatid gap (4.59), iso-chromatid gap (4.29) and centromeric dissociation (4.27). Moreover, the χ^2 value for this treated series are, 43.66 for chromatid break, 50.54 for subchromatid gap, 41.43 for centromeric dissociation, 20.32 for isochromatid break, 19.26 for rabbit-ear chromosome, 32.88 for rabbit-ear chromosome with gap and 48.30 for trans-location. Overall the χ^2 value is 188.94 for the pooled data. All these values are highly significant (up to 1% level). Overall, F-value for 2.4 Gy γ rays treated series is 10.69 and DMR value is 19.89 which are highly significant (Table 4).

It has been observed that 2.4 Gy γ rays were able to induce physiological changes in *M. musculus.* In the mice, when dissected after 16 h of whole body γ irradiation, it was found that abnormal excessive peritoneal fluid had accumulated in the stomach of one mouse (Fig. 13).

Moreover, after 1 month of treatment with the same dose of γ rays, a tumour (1 cm/0.5 cm) was observed between 2 eyes above the nose region in one mouse (Fig. 14).

DISCUSSION

Kihlman (1966) reported nonrandom distribution of chromatid breaks in mouse treated by chemicals. Jagetia et al. (2003) reported in mouse that naringin, a citrus flavonone is able to scavenge free radicals and thus could somewhat protect against γ radiation-induced chromosome damage. Mantena et al. (2008) reported radiation-induced aberrant metaphases and micro–nucleated erythrocytes at 24 h post exposure to 4 Gy γ radiations. Lemon et al. (2008) reported 2 Gy γ radiation DNA damage in whole body exposed normal and transgenic mice.

Sur et al. (2012a, 2012b) assessed the effect of 2 different doses of X-rays (0.8 Gy and 1.2 Gy) on bone marrow cells of mice. The present investigation reveals that the dose, 2.40 Gy is more genotoxic to mice than 0.8 Gy. In addition, it is found that the aberrations are nonrandom in distribution and are somewhat time dependent and centromeric regions of the chromosomes are most vulnerable to γ irradiation (Tables 2–5, Figs 1–12).

Whereas in *Suncus murinus* only 7 types of aberrations were observed when treated with 0.8 Gy and 2.4 Gy γ rays (Das 2023), in *M. musculus*, additionally, 2 more aberrations, rabbit-ear chromosome and rabbit-ear chromosome with gap have been observed. Moreover, the γ rays were able to induce maximum chromosomal aberrations after 16 h of exposure in shrews (9.02 % with 0.8 Gy and 12.38 % with 2.4 Gy), but in the present study with mice, maximum chromosomal aberrations is observed after 48 h of exposure (11.66% with 0.8 Gy and 19.01% with 2.4 Gy). In both the studies, 2.4 Gy γ irradiation is more genotoxic than 0.8 Gy, substantiating the fact that the effect of these radiations is dose dependent in nature. Even 2.40 Gy dose of γ rays is also observed to induce sub-lethal physiological changes in mice than 0.8 Gy (Figs 13–14).

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RESEARCH ARTICLE

TEMPERATURE AND METAL-STRESS INDUCED DNA DAMAGE AND HISTO-ARCHITECTURAL CHANGES IN EARTHWORM *LAMPITO MAURITII*

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SUMMARY In this study, DNA damage was assessed in coelomocytes of earthworm, *Lampito mauritii* upon exposure to chromium (Cr^{6+})-spiked soils at variable temperatures viz., 18° C, 24° C and 28° C to measure modulatory actions of temperature stress. Cr^{6+} co-exposures showed consistent percentage tail damage at all exposed temperatures. Histo-architectural changes in body wall and gut was studied to assess the impact of temperature and heavy metal stress at tissue level damage by exposing them to sub-lethal doses of chromium (Cr) for 30 d. The soil was spiked with Cr in laboratory conditions at 3 different temperatures of 18° C, 24° C and 28° C. Results revealed highly vacuolated epidermis with enormous swelling due to mucous gland proliferation and vacuole degeneration. Distortion of circular muscles were the major changes noticed in the body wall. Reduction and degeneration of chloragogenous tissue was observed resulting in a loss of cyto-architecture followed by its depletion. Altered shape and structure of intestinal epithelium with fused villi was prominent. Combined effect of temperature and metal resulted in vasodialation ensuing increased blood flow. Hence, the combined stress was more toxic and deleterious as compared to metal alone highlighting the synergistic effect. Lower temperatures were found more conducive for proliferation of gut epithelium when compared to higher temperatures. Cr severely affected the earthworm body wall as compared to gut. Thus, temperatures were an additional stress along with chromium and have shown alterations in all the tissues studied. Hence, DNA damage and histopathological alterations in *L. mauritii* can be considered as an important endpoint for toxicity evaluation and risk assessment of metal contaminated areas.

Keywords: DNA damage, chromium, comet assay, histo-architectural changes, temperature, metal stress, earthworm.

INTRODUCTION

Earthworms are essential organisms in soil toxicity testing, assessing environmental impact from heavy metal pollution (Rodriguez-Castellanos & Sanchez-Hernandez 2007). They require efficient detoxification systems for

survival due to continuous exposure to soil chemicals. Biomarkers in earthworms include toxic effects on reproduction, lysosomal stability, immune system, coelomocyte function, heat shock protein induction, DNA alterations and gene expression. Earthworm coelomocytes involved in internal defense, are widely used as non-destructive pollution biomarkers (Cooper et al. 2002, Engelmann et al. 2004, Semple & Martin 2017). Studies have shown DNA damage in earthworm coelomocytes after exposure to toxic chemicals such as pesticides (Xiao et al. 2006), imidacloprid (Wang et al. 2016), heavy metals (Cigerci et al. 2018), polycyclic aromatic hydrocarbons (Vernile et al. 2013), organic compounds (Sforzini et al. 2010) and radiation (Sowmithra et al. 2015). The efficacy of histological lesions as susceptible and consistent indicators of organism's health in their natural habitat (Otitoloju et al. 2009) and relation between xenobiotic exposure and development of toxicopathic hepatic lesions (Vethaak et al. 1996) has been established. Hence, stress-induced histopathological alterations are increasingly used as biomarkers of environmental stress (Stentiford & Feist 2005).

Perusal of literature has revealed histological changes in body wall and gut of earthworms on exposure to hexavalent Cr (Fernando et al. 2015), hydrocarbon induced stress (Eseigbe 2012), heavy metals (Vishal & Satyanarayan 2011) and herbicide glyphosate (Morowati 2002) using any single species of earthworm. In order to mimic the field conditions where multiple species prevail in a particular area, use of different species and multiple stressors for soil assessment is recommended (Van Straalen 2002). In this regard, to the best of our knowledge, no study has been reported so far on the combined effect of temperature and heavy metal stress on histological changes and DNA damage at different temperatures. Chromium is a heavy metal that exists in trivalent form, toxic at high doses (Rangaswamy et al. 2013) and hexavalent Cr is highly toxic at lower concentrations (Sivakumar & Subbhuraam 2005) and hence, it is important to study its impact on earthworms. The aim of this study is to report histological changes and DNA damage due to combined impact of temperature and heavy metal (chromium) on anecic earthworm *Lampito mauritii*.

MATERIAL AND METHODS

L. mauritii, the most abundant species reported from Bengaluru, was collected from a site with no history of the influx of pollutants. Worms were carefully transported and accli-matized for one month under laboratory conditions in plastic culture boxes containing soil and farmyard manure mixture (3:1) with a moisture content of 35–40% and pH 6.8 ± 7.0. Earthworms used in this study were mature with a well developed clitellum. They were picked from the culture boxes 24 h prior to use and left in petri dishes on damp filter paper in the dark at 28 ± 2° C to void their gut contents. The individual's fresh weight ranges from 400–600 mg.

As per available literature, the concentration of total Cr reported in Bengaluru soils were below the permissible limit of 0.1–0.3 ppm. In effluents,

the permissible limit of Cr is up to 2 ppm, as prescribed by Central Pollution Control Board, India. The 14 d LC 50 values for Cr was 16.57 ppm (R^2 = 0.9601) derived from Finney's probit analysis (Finney 1971). In view of the above ranges, a sub-lethal dose of 8 ppm was used in the present study.

Experimental beds were created by mixing fine sieved soil and sun-dried cow dung powder in a plastic culture box. Potassium dichromate solution was added to the soil to attain required concentration of 8 ppm Cr, and moisture content was adjusted to 30% of the final weight of soil and allowed to stabilize for a wk. Control beds were prepared using distilled water, and 10 gut-evacuated earthworms were added to each bed. Metal exposure replicates were maintained at a different temperatures of 18° C, 25° C and 28° C, and 4 worms were randomly picked for further processing.

Coelomocytes from earthworms were isolated by using a non-invasive extrusion method of Eyambe et al. (1991). Coelomocytes were washed with lumbricus balanced solution and centrifuged at 3000 rpm for 10 min. The cell count and viability were checked using a haemocytometer and tryphan blue dye. Samples with over 90% viability and a cell count of 10^6 cells/ml were used for the comet assay (Sowmithra et al. 2015).

The comet assay was conducted using the method of Singh et al. (1988), with slight modifications. The cell suspension was mixed with 0.7% low melting agarose, pipetted onto slides, and then immersed in a lysis solution. Triton X-100 was added before electrophoresis.

DNA unwinding was achieved by incubating slides in electrophoretic buffer, electrophoresing at 25 V and 300 mA, neutralizing and fixing in anhydrous ethanol. Slides were stained with ethidium bromide and 50 cells per slide were scored to assess comet status and images were analyzed to determine DNA damage. Percentage change was calculated using the following formula:

$$\text{Percentage change} = \frac{\text{Control} - \text{Experimental group}}{\text{Control}} \times 100$$

Four earthworms from each culture box were removed randomly after 30 d of metal exposure and left on the moist filter paper for removal of gut contents. Later, the animals were dissected and small pieces of the intestine were removed and fixed in Bouin's fixative for 24 h. Thereafter, the tissue was washed, dehydrated, cleared and embedded in paraffin wax. Embedded tissues were sectioned at 3 mm thick and spread on slides coated with albumin. Sections were stained with haematoxylin and eosin, dehydrated and mounted with DPX.

OBSERVATIONS

Upon exposure to temperature stress alone at 18° C, an augmentation (+136.17%) in tail DNA was evident. Exposures at 28°C revealed a severe exacerbation (+375.08%) in tail DNA representing

significant DNA damage. Exposures to combined stress of Cr^{6+} and temperature revealed a severe exacerbation in tail DNA and the observed changes were found to be +166.59% at 18° C and 181.07% at 28° C highlighting severe DNA damage in comparison with control at 24° C (Figs 2–4).

In the body wall of *L. mauritii,* at 18° C, considerable epidermal glandular proliferation in Cr-spiked soils was observed. On continuous exposure to 30 d, change in shape of circular muscle fibres was seen in temperature exposed group alone but in Cr-spiked soils at 18° C distortion of epidermal layer was prominent (Figs 5–10).

In the gut, complete fusion of villi and disappearance of chloragogen tissue was prominent in Cr-spiked soils. At 24° C, damage to epidermal layer and proliferation of chloragogenous tissue was observed upon 15 d of exposure. On the contrary, after 30 d of exposure, intercellular spaces between the villi and vacuole formation in chloragogen tissue was evident in Cr-spiked soils. At 28° C, in Cr-spiked soils, severe mutilation of both circular and longitudinal muscle layer followed by epidermal distortion was observed. In the gut, fading of chloragogenous tissue layer and its evasion was seen on exposure to temperature alone but in Cr-spiked soils, thinning of chloragogenous layer was observed on 15 d of exposure and on prolonged 30 d of exposure to Cr, fading of the layer was observed (Figs 11–16).

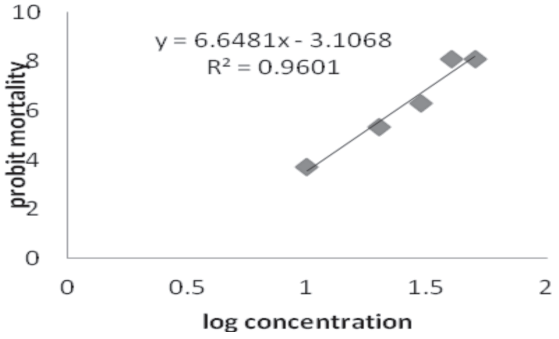
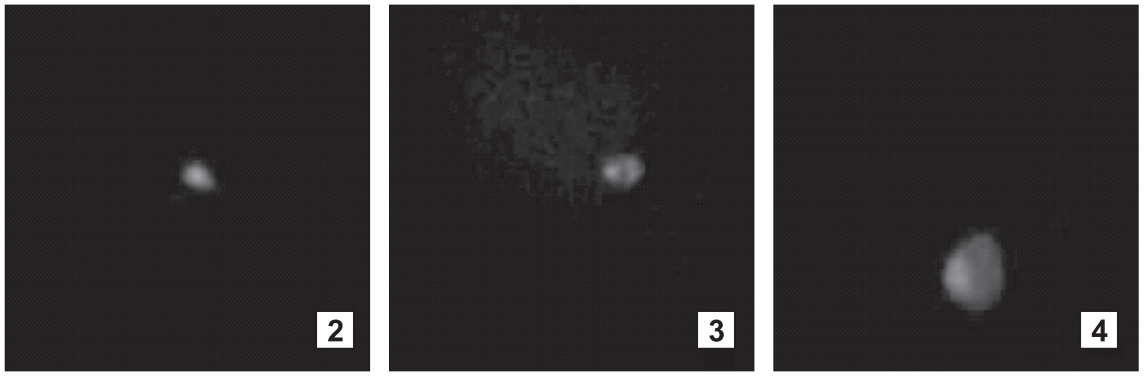
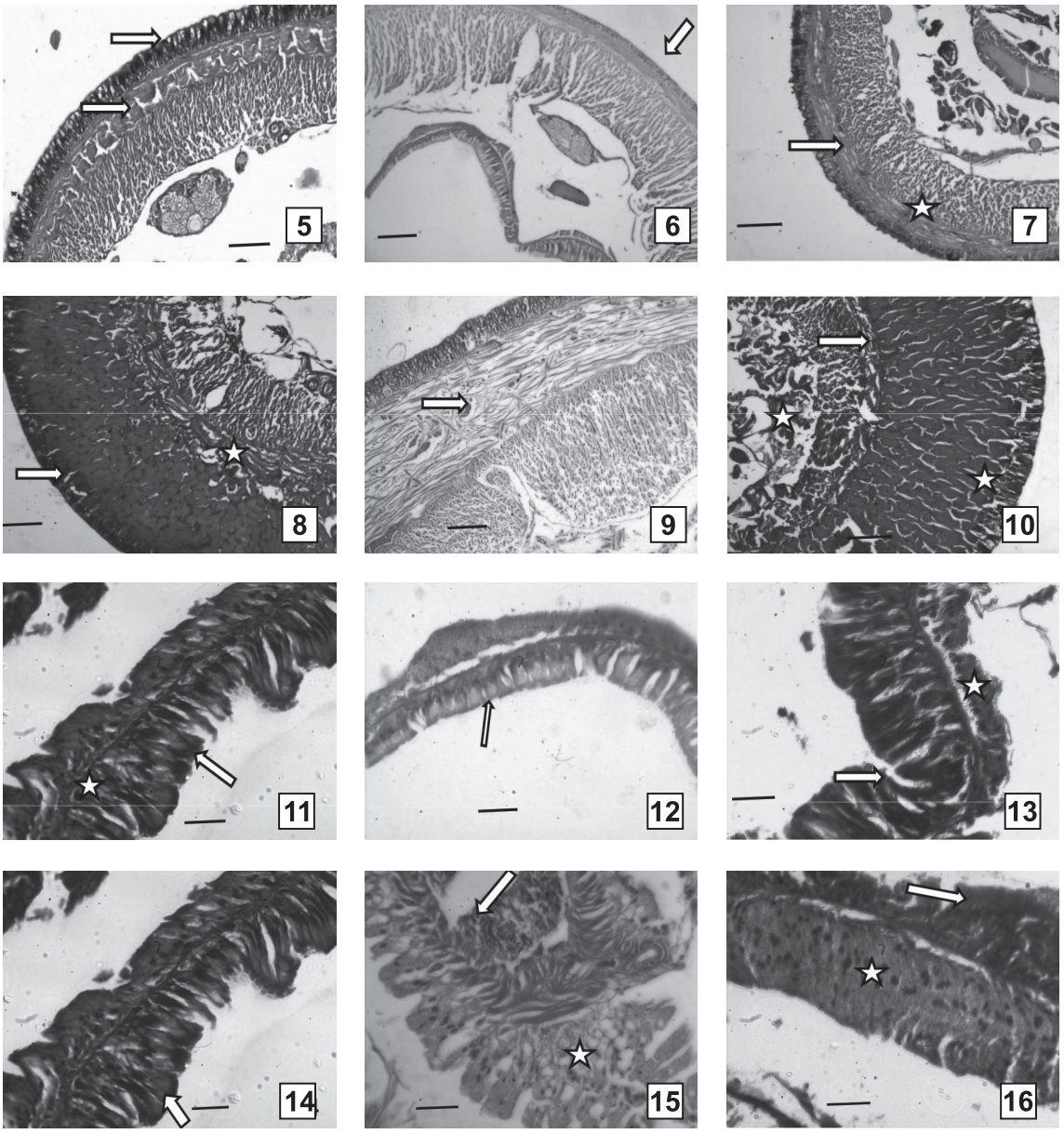


Fig. 1: 14 d probit mortality of *L. mauritii* exposed to different concentrations of Cr.



Figs 2–4: DNA strand breaks (% tail DNA) in *L. mauritii* upon co-exposure to sublethal doses of Cr (8 ppm) and temperature stress. 2. Coelomocytes showing DNA damage at 18° C. 3. Coelomocytes showing DNA damage at 24° C. 4. Coelomocytes showing DNA damage at 28° C.



Figs 5–16: Histological changes observed in the body wall and gut of *L. mauritii* upon exposure to temperature and combined stress. 5–10. Body wall showing epidermal glandular proliferation and mutilations in longitudinal and circular muscle fibres (arrows indicate glandular proliferation, star indicates mutilation of muscle fibres). 11–16. Changes in the gut showing fusion of villi and fading of chloragogenous tissue. 15,16. Intercellular spaces between the villi and vacuole formation in chloragogenous tissue (arrows indicate fusion of villi, star indicates vacuole formation). Scale bar = 10 μm.

DISCUSSION

Understanding susceptibility to DNA damage in coelomocytes of earthworms presumed to be useful in understanding the overall impact of heavy metals. In addition, the main goal of the study was to understand the response of earthworms to temperature stress. The worms were acclimatized by changing temperature (1° C/day) for 6 d, earthworms were then exposed to temperature conditions of 18° C (cold), 24° C (control), and 28° C (warm) over a 30 d period. The DNA damage witnessed could be base lesions, strand cross links (intra and inter), DNA–protein cross–links and both single and double strand breaks. In the present study, existence of tail DNA damage observed in the control group of earthworms reflect the natural DNA damage or elimination of damaged cells by apoptosis. Similar observations have been reported by Collins (2004). These observed DNA damage correlates with histological alterations. The changes observed in the earthworm's body wall may be due to the exposure to metals in soils as the skin is the major route of pollutants uptake. Previous studies suggested that earthworm's skin has direct contact with contaminated soils and is considered as a significant route of uptake of toxicants (Vijver et al. 2003). Likewise, excessive epidermal mucous secretion as a result of contact with pollutants was observed by Rao et al. (2003) and Pan et al. (2010). The severe changes in the body wall of earthworm in the temperature exposed groups may be due to the accumulated metals in the body wall. Kilic (2011) reported accumulation of metals in circular and longitudinal muscles of animals in polluted soils. The same may be the reason for the observed changes in the circular and longitudinal muscles of the body wall of the worms. Upon continued exposure at higher temperatures, cells have completely lost their shape resulting in loss of cytoarchitecture followed by cell necrosis. Atrophy of the intestinal epithelium was clear with fused villi forming continuous and thin layer with poor staining ability. Similar observations were made by Kilic (2011) on exposing earthworms to soils polluted with metals and radionuclide. Further, Ware (2000) reported alterations in chloragogenous cells upon fluoride exposure. The chloragogenous tissue, which is a sheath of modified peritoneal cells, in gastro-intestinal canal and separating the absorptive epithelium (villi) from the coelom, constitutes the main site of metal accumulation. According to Morgan et al. (2002) morphological alterations in the earthworm's chloragogenous tissue was a means of handling larger quantities of metals by extrusion of whole chloragocytes, which enables earthworms to tolerate high concentrations of metals in the soil (Langdon et al. 1999, 2001). Increased mucous production in the intestinal tract may play an important role in breakdown of organic macromolecules (Double & Brown 1998) which is known to induce swelling, necrosis and intestinal pathology in earthworm species. Morgan et al. (2002) reported on the metal accumulation

capacity of intestinal epithelium in an Oligocheate, *Dendrodrilus rubidus*. The changes observed in this study can also be attributed to functionality of the chloragogenous tissue which got evaded in order to cope up with the metal stress by releasing chloragocytes into the coelom.

It can be inferred from the present findings that DNA alterations have the potential to predict deleterious effects in exposed species alongside the histological details. Thereby, the combination of comet assay representing genotoxicity in coelomocytes and histo-architectural changes can be considered as a warning signal of sublethal doses of metal stress and interactive effects of temperature in earthworms, as it closely represents the field conditions in environmental biomonitoring studies.

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