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Full Length Research Paper

Phorate poisoning of a leopard (*Panthera pardus*) in the Nilgiris

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India is an agricultural country; animal husbandry has always been associated with agriculture. People still thrive upon animal products such as milk, meat and manure intensely for their essentialities. India has a huge cattle population and most of them graze in areas close to forests and their fringes competing for their pastoral needs with other wild animals. This then leads to human-wildlife conflicts, which tends to culminate in a number of tragic outcomes, including wild animal poisoning. Poisoning is perceived as an easy way for people to rid themselves of wild animals. Numerous factors, including the type of agriculture practices conducted, public knowledge regarding toxicity of a specific product, cost, availability in the local market place and physical properties such as color, taste and odor determine the extent to which specific pesticides are used to deliberately poison wild animals. This paper deals with a case of phorate poisoning, which is an agrochemical, in a leopard in Sholerock Estate, Coonoor, Nilgiris district. An empty sachet of phorate was found close by. This was confirmed by the result from Regional Forensic Science Laboratory (RFSL). Leopard is however more versatile and can adapt to diverse conditions. It is often observed within the core and in the buffer zones surrounding protected areas and managed forests. It can tolerate human presence to a point. There have been several incidences where leopards have preyed on livestock, dogs, children and even adult humans leading to conflict. Therefore, the loss of an apex predator, that holds a significant position in the upper trophic level, will have deleterious effect on the balance, ultimately threatening human survival directly and indirectly.

Key words: Conflict, phorate, poisoning.

INTRODUCTION

Life in the Nilgiris has always had its part and parcels with wildlife either as a boon or a bane. It is just in these recent years that the scenarios of human wildlife conflicts have taken a toll adversely affecting the normal life patterns of people and wild animals. Wild animals often come into conflict with people by destroying agricultural crops and even killing people, thus providing a deterrent to conservation efforts. Further human settlements had a dual impact on wild animal-habitat. The directly visible and measurable one was that of habitat loss through conversion of wild animal's habitat to human use and the second is an indirect human influence and impact on wild

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animal habitat. Human settlements were almost in close proximity to water. Areas close to water were the preferred areas for wild animals and loss of such areas has a much more serious impact. Thus human settlements not only deprived the wild animals of the use of significantly large areas of habitat, but also deprived them of significantly preferred habitat. Human-wildlife conflicts were found to be intensified as population growth forced the development activities which infringed on wildlife habitats. This led to fragmentation and declining of habitat quality, eventually causing competitions between humans and various wildlife species with regard to space and resources and stressed wild animals often turned to crops or livestock for food.

Many domestic animals also live on the forest fringes, competing for pasture with wild animals, leading to human wild life conflicts which tend to culminate ultimately in a number of tragic outcomes, including wild animal poisoning. In conflict areas, large carnivores were often the primary targets for malicious poisoning and they are wiped out giving a temporary relief, unknowingly leading to an intense biological imbalance causing a catastrophe in the food web.

MATERIALS AND METHODS

The veterinary surgeon of Selas, Coonoor town, Nilgiris district was informed about the presence of the carcass of a female leopard in his jurisdiction. The location of the carcass was amidst the dense tea bushes with a small stream outlaying closely. A detailed necropsy procedure was conducted. A composite sample (300 g) containing stomach and intestinal loop with contents separately, together with portions of liver, kidney, lung, and heart were collected separately in 500 ml of saturated sodium chloride in sealed containers from the leopard carcass in saturated sodium chloride solution as preservative and sent along with plain preservative as control to the Regional Forensic Science Laboratory (RFSL), Coimbatore, Government of Tamil Nadu, for toxicological analysis. The samples were examined and analyzed using thin-layer chromatography followed by gas chromatography–mass spectrophotometry at RFSL. On walking back to the road, an empty sachet of insecticide inscribed phorate (10%) was retrieved approximately 300 m away from the carcass. Environmental findings led to the suspicion of phorate poisoning. The ancillary trace evidence (Phorate 10% cover) was collected and retained for analysis. Maintaining chain of custody of the collected evidence was done by the forest department; the Squad ranger of the particular range was entitled the duty of sample delivery and result follow up.

RESULTS

Necropsy was done in a systematic manner. Externally, there was no characteristic lesion or striking abnormality. Internally there was generalized congestion and hemorrhage. The oral cavity was inspected for any material of meat shreds trapped in the mucosal folds. The teeth were all intact, the gingivae showed congestive changes. The visible mucous membrane was brick red in color. The peritoneal cavity contained sanguineous fluid (haemoperitoneum). There were hemorrhage and congestion on the trachea and the lung showed severe congestion and was edematous. The pericardium was filled with sero-sanguineous fluid and the epicardium and endocardium exhibited petichae. A generalized garlic odour was prevalent throughout the procedure. The stomach contained about 500 to 1000 g of partially digested material and with slurry of flesh and lots of hair, bristles and some semi digested bones. The mucosa of the stomach showed petichae and the intestinal mucosa were congested. There was generalized congestion and hemorrhage. The kidneys revealed sub capsular hemorrhage. The shape of the spleen was altered and was dark red in color. These recordings were in agreement with the findings recorded by Kalaivanan et al. (2010). Typical lesions of toxicity like pulmonary edema, hemorrhagic intestinal tracts, necrotic and degenerative changes in the liver and kidney were evident. The result from the RFSL proved that about 4.7 g percent of phorate equivalent was estimated from the composite sample.

The necropsy findings, circumstantial evidence and laboratory confirmations strongly suggest that the reason for death was due to poisoning by organo phosphorus compound (OPC) indicatively phorate.

DISCUSSION

The common uses of phorate for the tea plantations and to the adjoining agricultural cultivation were found out. The general agricultural practices pertaining to the Nilgiris include cultivation of carrots, potatoes, cabbages and lots of exotic vegetables. The package of practices includes the use of agro chemicals such as phorates, carbamates, endosulfans and malathion in general to their fields. The minimum toxic dose of phorate that was found to be toxic in domestic animals ranged from 0.25 to 1.00 mg/kg (Tiwari and Sinha, 2010). A very interesting fact was that the damages that these people faced by the crop raiding by the wild pigs in this region and the adjoining regions was least tolerated as nearly 50% of the profit was lost on curbing wild pigs (Gopakumar et al., 2012). The availability of the highly palatable feed varieties, increase in predator-prey density; increase in competition among the co-existing herbivores and omnivores in the adjoining forest region, carrying capacity of the region, extensive activities or manipulation by human beings in the forest regions have made wild pigs an agricultural pest; this was in agreement with findings made by Chauhan et al. (2009). In this context, poisoning wild pigs using agro chemicals was conflict mitigation strategy in these regions. Usually potatoes laced with poisons or commercial broilers stuffed with toxic compounds have been found to be positioned on the tracts of the wild pigs, luring them into taking up the toxin. However, leopards and tigers also share the same landscape and often be-

come accidental targets ending down the apex predator that occupies the topmost level in the food chain. This in turn reflects very badly as the increase in the predator quotient and the prey like wild pigs supervene and cause excessive damage than a higher level that was prevailing. Therefore, what was supposed to be a conflict mitigation strategy ended up increasing the damage.

The use of agro chemicals for attributable crop growth becomes inevitable in our country, but this serves as a blessing in disguise for a lot of defaulters who take advantage of this and are unaware of the damage they are causing for sense of temporary relief. Hence, the management plan in the protected regions shall focus in the measures that help to prevent the deterioration of feed-resources for the wild pigs. Similarly, appropriate crop insurance schemes might be strengthened pertaining to the wild pig associated high risk croplands identified and all these might definitely help to mitigate the conflict problems between the wild pigs and humans in the areas adjoining the wildlife region, thereby preventing the break in the food chain and ultimately maintaining balance.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Full Length Research Paper

Evidence of micronuclei in fish blood as a biomarker of genotoxicity due to surface run off agricultural fungicide (Propiconazole)

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Micronuclei, nucleoplasmic bridges and nuclear buds are biomarkers of genotoxic events and chromosomal instability. In laboratory, these genome damages can be measured easily. The measurement of cytogenetic alterations *in-vivo* has considered an initial step in the risk assessment procedures by genotoxic agents. In fishes, micronucleus assay has shown useful *in vivo* techniques for genotoxicity testing, and potential for *in situ* monitoring of water quality. This paper evaluates the genotoxic effects of fungicide in fish erythrocytes, with emphasis on the induction of micronuclei formation. The binucleate/mononucleate cells ratio in peripheral erythrocytes exposed to propiconazole (1.11 and 2.23 mg/L) has also been used to evaluate the time-dependent response. Micronucleus frequencies induced by fungicide is significantly greater than their respective control ($p < 0.05$) for the fish species *Clarias batrachus* throughout all treatment periods. This paper is directed to assisting laboratories in the development of micronucleus test for assays of genotoxic potential of chemicals.

Key words: Micronucleus assay, propiconazole, genotoxicity, binucleate/mononucleate cells.

INTRODUCTION

Propiconazole (PCZ) is a trizole group fungicides which was introduced in mid 1970s classified as category III and IV. Trizole containing fungicides have been used as an antifungal in agriculture for controling pest and also for increasing food crops (Vanden Bossche et al., 1989; Warmerdam, 2008). Trizole, belonging to demethylation inhibitors (DMI) group with rapid acropetal systemicity acts on the pathogen inside the plant to stop disease development by interfering with sterol biosynthesis in fungal cell membrane. The trizole fungicides inhibit one specific enzyme C14-demethylase, which plays a role in sterol production. It has a shorter half-life and lower bioaccumulation, but announcing effects on the aquatic ecosystems may arise from spray drift or surface run-off (Konwick et al., 2006). It has been reported that it undergoes transformation of secondary metabolites in terrestrial mammals (Chen et al., 2008). Series of studies show that the trizole fungicides alter the metabolic pathways, cell signaling, cell growth pathways, cell cycle genes and other transcriptional factors (Bruno et al., 2009; Nesnow et al., 2009; Li et al., 2010). In body tissues, it metabolite into 1,2,4 tizole, trizole alanine, trizole acetic acids, trizole pyruvic acid and trizole lactic acid. Of these metabolites,1,2,4 trizole and trizole alanine

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are the main metabolites which have toxic effects. 1,2,4 trizole is an isomeric chemical compound which have 5-membered ring of 2 C-atom and 3 N-atom. Due to the presence of lone pair electron on N_2-atom, it has specific toxicity. Therefore, because of its widespread use, toxicity evaluations have been performed in this studies.

In aquatic organisms, the micronucleus test (MNT) is one of the most applicable techniques for identifying the genomic alterations. This assay targets interphase cells of any proliferating cell population regardless of its karyotype. In environmental biomonitoring programme, this technique has been adapting because of its simplicity. The purpose of this paper is to know, whether the used fungicide has genotoxic property or not in reference to aquatic organisms.

There are several events of formation of micronuclei (MN) and other nuclear lesions in blood such as fragments caused by mis-repair of DNA breaks or unrepaired DNA breaks or mal-segregation of whole chromosomes at anaphase, hypo-methylation of repeat sequences in centromeric and peri-centromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes, dysfunction in the error frees homologous recombination DNA repair pathways and defect in enzymes of non-homologous end joining pathways in organisms (Fenech, 2007; Fenech and Crott, 2002; O'Donovan and Livingston, 2010; Srivastava and Singh, 2014).

Other important mechanisms, which induce MN formation in blood of acentric fragments, include simultaneous excision repair of damage or inappropriate base incorporated in complementary DNA strands (e.g., uracil) (Fenech and Crott, 2002).

Irrespective of the aforementioned reports, the reasonable mechanisms that lead to MN formation is not yet complete. In genomics, the specific gene expression patterns responsible with the formation of MN, is still rudimentary (Iarmarcovai et al., 2008). In the present study, the formations of MN frequencies have been studied with the exposure of fungicide, PCZ.

MATERIALS AND METHODS

PCZ (CAS No. 60207-90-1) has been purchased from Syngenta Ltd. from India, a technical grade pesticide. Other chemicals such as Giemsa stain has been purchased from Indian market. Freshwater fish *Clarias batrachus* were obtained from local hatchery (Chhappy Hatchery). Fish had an average weight of 40.01 g and average length 17.26 cm. Fish were fed with commercial fish food, and acclimatized under laboratory conditions for 2 weeks, containing de-chorinated tap water (pH = 7.6 alkalinity, 150 mg/L $CaCO_3$, dissolve oxygen (DO) = 7.03 mg/L, temperature = 22°C). The photoperiod used 12 h/12 h dark/light. Aquaria used in study was made of glass and had constant aeration. Physical dimensions of aquarium had 100×40×40 cm, and a 120 L capacity.

Fishes were divided into 4 groups and each group containing 10 individual fishes. Group1: negative control (only tap water was used), Group 2: positive control (4 mg/L cyclophosphamide; Sigma), Group 3: 1.11 mg/L PCZ, Group 4: 2.23 mg/L PCZ according to pre test (Srivastava and Singh, 2013).

Blood smear slides were prepare by the method of Das and Nanda (1986) with some modifications. Peripheral blood samples were obtained from caudal vein of fish from 24, 48, 72 and 96 h. Blood smears were prepared from each group of randomly selected fishes. At each assessment, 2500 cell/fish were analyzed (7500 erythrocytes for each group). Slides just dried in air, fixed in absolute methanol for 10 to 15 min, and stained in Giemsa (pH 7.0) for 1 to 2 h. The average frequencies of abnormalities were determined for each group as presented in Table 1. MN frequencies (MN‰) were calculated as follows:

$$\text{MN (‰)} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 1000$$

Then the mean ± standard error for each group was calculated. Student's t-test was employed for comparison of control and experimental animals (Sokal and Rohlf, 1973).

RESULTS

The results of micronucleus and nuclear abnormalities in peripheral erythrocytes of *C. batrachus* is as shown in Table 1 and Figure 1. In both groups which were receiving fungicide as well as in the positive control group, frequencies of MN and other nuclear abnormalities were observed significantly higher $p<0.05$ when compared with negative control group. In Group 3, which was receiving 1.11 mg/L PCZ, increased frequencies of MN were found in erythrocyte after 48 h in comparison to 24, 72 and 96 h with respect to negative control. Group 4, receiving 2.23 mg/L, showed the same results in all periods, but the frequencies of MN and other nuclear anomalies were higher than Group 3 fishes. In positive control group, the results were also similar. In both Groups 3 and 4 and also in positive control, the frequencies of lobbed nuclei (LN), notched nuclei (NN), nuclear buds (NB) and nucleoplasmic bridge (NPB) were found to significantly increased after 24 and 48 h. NN and NPB increased after 24 and LN and NB increased after 48 h (Table 1).

DISCUSSION

The results of the present study show the induction of MN and other nuclear anomalies in peripheral erythrocytes of *C. batrachus* when exposed to different concentrations of

Table 1. Frequencies of formation of micronuclei and nuclear anomalies.

Assay	Exposure period	Group 1	Group 2	Group 3	Group 4
		Average frequency	Average frequency	Average frequency	Average frequency
NN	24 h	1.06±0.03	8.0±0.12*	5.2±0.10*	6.8±0.12*
LN	”	0.66±0.009	8.2±0.12*	4.93±0.09*	6.53±0.11*
NB	”	0.66±0.009	9.06±0.14*	4.4±0.07*	7.6±0.13*
NPB	”	0.93±0.02	6.9±0.11*	3.86±0.06*	4.93±0.09*
Micronuclei	”	0.66±0.009	34.8±0.25*	23.46±0.20*	30.13±0.25*
NN	48 h	0.66±0.009	6.8±0.11*	4.26±0.06*	5.2±0.07*
LN	”	1.46±0.04	11.33±0.15*	8.13±0.12*	9.2±0.14*
NB	”	1.46±0.04	12.26±0.16*	6.0±0.10*	10.93±0.15*
NPB	”	0.66±0.009	5.6±0.10*	3.33±0.05*	4.8±0.06*
Micronuclei	”	1.46±0.04	42.4±0.30*	32.0±0.27*	34.93±0.30*
NN	72 h	0.68±0.01	4.53±0.08*	2.86±0.04*	2.66±0.04*
LN	”	0.93±0.02	7.33±0.10*	5.06±0.07*	5.46±0.08*
NB	”	0.94±0.02	7.46±0.10*	3.73±0.07*	5.06±0.08*
NPB	”	0.4±0.006	4.93±0.09*	1.73±0.05*	2.53±0.07*
Micronuclei	”	0.93±0.02	35.2±0.25*	21.73±0.18*	24.66±0.21*
NN	96 h	0.67±0.01	2.93±0.08*	1.33±0.03*	1.46±0.04*
LN	”	0.66±0.009	5.86±0.10*	3.33±0.06*	3.86±0.05*
NB	”	0.94±0.02	6.93±0.11*	3.20±0.04*	4.66±0.06*
NPB	”	0.4±0.006	4.66±0.08*	1.33±0.03*	2.13±0.06*
Micronuclei	”	0.67±0.01	32.8±0.22*	17.86±0.17*	20.86±0.20*

*$p < 0.05$ Significant value. NN: Notched nuclei, LN: Lobed nuclei, NB: Nuclear bud, NPB: Nucleoplasmic bridge.

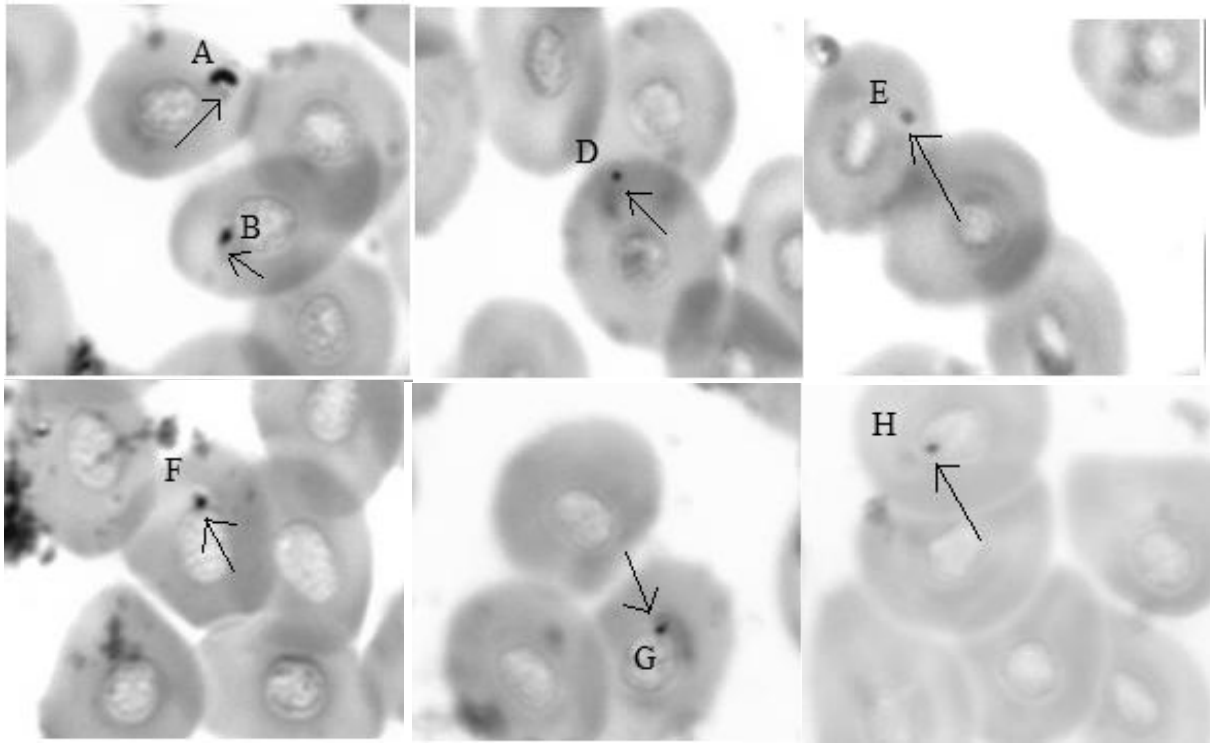

Figure 1. Micronuclei (MN) in blood of fish *Clarias batrachus* after treatment with Propiconazole. A = notched nuclei, B, F, H, and G = lobed nuclei, and D and E= micronuclei.

fungicide PCZ. PCZ is a trizole group fungicide. It has 1H-1,2,4-trizole group at 4-position, 1,3-dioxolane moiety and an aryloxymethyl group and also nitrogen containing hetero-aromatic ring. In blood and tissue, it metabolites into 1, 2,4-trizole alanine, is the main metabolite of trizole containing fungicides. Metabolites of trizole also produce many N_2-containing substances that might be affected on enzymes activity and also nucleic acids (Srivastava and Singh, 2013).

Increasing concentration of nitrogenous metabolites in blood affects the series of enzymatic systems in fish and induces the formation of reactive oxygen species (ROS). Production of reactive intermediates such as ROS, are highly toxic cause DNA lesion in fish blood (Pandey et al., 2011). DNA lesion can be different in shape and size such as in bud form (attached to the main nucleus), lobed form (evagination of nuclear membrane), or in bridge form, however, each anomalies are the signal of cytogenetic damages in fish that could be resulted with failures in cell division, cell death process with mutagenecity (Bombail et al., 2001; Pachecco and Santos, 2002).

After different exposures of fungicide, the formation of MN, NB, NPB and other anomalies were observed. It has been suggested that gene amplification via the breakage-fusion-bridge cycle could cause formation of nuclear anomalies during the elimination of amplified DNA from the nucleus (Shimizu et al., 1998). Abnormal cell division due to the blocking of cytokinesis can also cause binucleation that may lead genetic imbalance in the blood (Rodilla, 1993). Formation of these anomalies in fish blood clearly indicate that PCZ has high genotoxic potency and insight of socio-economic purpose; it is very harmful for aquatic ecosystem. However, the well-documented and reasonable significant associations with an increase in the occurrence of MN and exposure periods are still unknown (Bolognesi et al., 2006). Although the significant increases in erythrocyte MN in fish after 48 h was recorded, whereas some workers have demonstrated the increased frequencies of MN at 72 h. These variations may also be a direct consequence of the sensitivity behavior and the niche of the species, as the number of MN in the cells of fish may be variable (Graziela et al., 2010). Due to chemical pollutants, the low amount of DNA per cell, the large numbers of small chromosomes and the low mitotic activity in many fish species impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges are demonstrated (Pavlica et al., 2000).

Conclusion

It is evident that multiple mechanisms can lead to the formation of MN. MN frequency provides a useful index of accumulated genetic damage during the lifespan of the cells. MN expression strongly depends on the mitotic activity in the studied tissue, which in turn depends on a complex interaction of biotic and environmental parameters such as temperature, salinity and food availability. The MN frequencies in blood evaluate the kinetics of cytogenetic alterations under fungicides influence. So based on the present study, it was concluded that the fungicide PCZ has great potential to alter the DNA model. Therefore, the extensive use of these fungicides in field crop and its surface run-off at near water bodies should be avoided.

Conflict of interest

The authors declare that there are no conflicts of interest.

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