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Potentiality of Short – Term Cultured Cells of Carps in DNA Damage and Repair Studies

G. Mohanty[1*] and J. Mohanty[2]

[1]Department of Zoology, North Orissa University, Baripada -757003, Orissa, India
[2]Central Institute of Freshwater Aquaculture, Kausalyaganga - Bhubaneswar -751002, Orissa, India
E-mail: [1]gargee.mohanty@gmail.com, [2]mohantyj@hotmail.com

Abstract—*Short-term blood cell culture was carried out to study the DNA damage and repair in rohu (Labeo rohita) exposed to an organophosphate pesticide, phorate. Twenty four hours cultured cells were exposed to 4, 20, 100 and 500 µg/ml concentrations of phorate for 3h. To evaluate the extent of DNA damage, cultured cells from three sets of control pertriplates and three sets of each treatment group were harvested separately and subjected to alkaline comet assay. Similarly sets in triplicates were transferred to pesticide free media and sampled at 1.5 and 3h of transfer. Results showed that pesticide was able to induce genotoxic effects after 3h of pesticide exposure. However after 1.5 h in the pesticide free media all the doses of the pesticide showed reduction in the number of DNA breaks. A complete repair of DNA breaks in 4 and 20 µg/ml concentration levels of phorate treated cells were noticed at 3h of incubation in the pesticide free media. The present study thus advocates the utility of in vitro system in the study of genotoxic potentialities of compounds on whole blood cultured cells of rohu owing to its capability to detect DNA damage as well as its repair. One of the important outcomes of our study is that the whole blood cells of carp like rohu can be cultured on short – term basis without the support of serum and carbon dioxide tension.*

Keywords*: Short - term cell culture, phorate, comet assay, DNA damage, DNA repair.*

1. INTRODUCTION

During the last decade, significant effort has been expanded in developing rapid toxicity assays. There has been increasing need to assess the toxicity of various samples types in minutes to hours instead of days. Many of the short term assays developed thus far are based upon biochemical, physiological, biochemical, molecular, genotoxicological, or histological markers of anthropogenic stress in place of survival, growth and reproduction. The measurements made during these assays that identify the causative agents of such changes in the body of the organisms are collectively called as biomarkers. The intent of these assays has been to provide shorter, easier to obtain measurements endpoints and to provide highly sensitive indicators of biological health and functions. Biomarker measurements reflect the bioavailability of contaminants, provide rapid toxicity assessment and an early indication of population and community stress, and offer scope to study the effects of specific chemicals and other harmful agents. Chemical effects are thought to be the result of interaction between toxicant and biochemical receptor. Therefore, biochemical responses should occur before effects are observed at higher level of organization. In natural environments, organisms are exposed to multiple stresses both natural occurring and chemical borne, that are integrated over time. The use of biomarkers should reflect these stresses and provide an early indication of the potential for the population level effects.

In order to assay the extent of mutagenicity or genotoxicity caused in the organisms of a population a number of of genotoxicity assays have been developed that include systems unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), single cell gel electrophoresis (SCGE) commonly called comet assay, formation of DNA adducts and mitotic recombination.

Pesticides are substances used to control pests, including insects and plant diseases. Naturally-occurring pesticides have been in use since centuries, but widespread production and use of modern synthetic pesticides did not begin until the 1940s. On a global scale approximately over five billion pounds of conventional pesticides are being in use in different areas like agricultural lands, forests, rangelands management, disease control, domestic use and many more areas annually[1].

Comet assay or single cell gel electrophoresis (SCGE), primarily detects any damage in DNA strands that basically includes single and double strand breaks, alkali labile lesions, oxidative damages and cross-linking activities of proteins with DNA. It has been widely applied as the first endpoint to determine genotoxicity in eukaryotic cells [2]. SCGE can be performed on any eukaryotic cells. The technique essentially does not need dividing cells [3-5]. Recently, comet assay has been widely used in studying DNA repair kinetics [2].

Traditionally, the environmental hazard on vertebrates in aquatic systems is evaluated by performing acute [6] and chronic fish experiments [6, 7]. Recently, there has been increased interest in replacing *in vivo* fish toxicity tests with *in*

vitro fish cell tests, with the aims to overcome the above disadvantages and reducing the use of live animals [8]. Moreover, the international recommendations for the reduction of the use of laboratory animals [9], has also emphasized upon the utility *in vitro* test systems. In the last decade, more than 150 continuous fish cell lines have been established and a number of bioassays have been developed in an attempt to replace acute toxicity tests with fish [9, 10]. Cell lines such as hepatoma cells PLHC-1 from the desert topminnow (*Poeciliopsis lucida)* [11-12], hepatocytes and gill epithelial cells from rainbow trout (*Oncorhynchus mykiss*) [8, 13-14] were shown to be suitable for the determination of cytotoxicity endpoints. The present study has thus been carried out to exemplify the use of comet assay and *in vitro* test systems for the DNA damage and repair kinetics studies in cultured cells of fish. In this study the short term cultures were maintained without the aid of serum and carbon dioxide tension which makes the cells more useful to carry out test related to different cytotoxicity, genotoxicity and many others.

2. MATERIALS AND METHODS

2.1. Test chemical

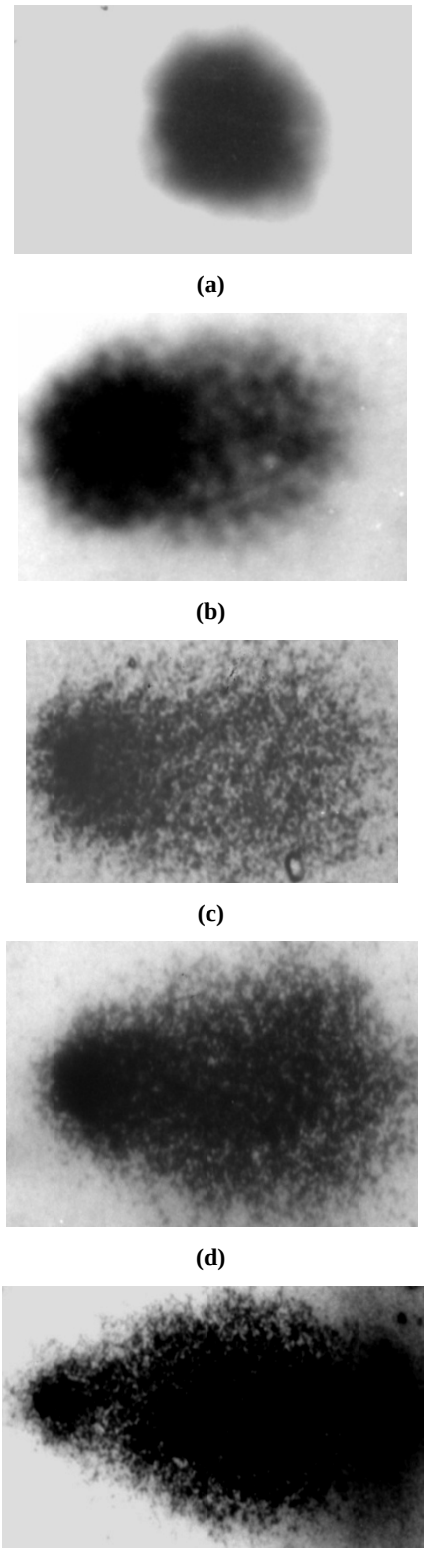
Phorate (10% CG, Trade name: Thimet, Agri More Private Limited, India), an organophosphate pesticide was procured from the local market. Low and normal melting agarose were bought from SRL, India. All other chemicals used were of analytical grade or higher. Cell culture media, balanced salt solutions were of purchased from Sigma Aldrich, USA.

2.2. Short term cultures of whole blood cells of rohu

A short-term whole blood cell culture was carried out according to the standard protocol of Freshney (2000). Blood from *Labeo rohita* fingerling was collected by cardiac puncture into an ACD (0.48 % w/v Citric acid, 1.32 % w/v, 1.47 % w/v D-Glucose) containing syringe in the laminar flow hood. Approximately 0.2 ml of blood was released into each sterile centrifuge tube and was mixed with 1.5 ml of sterile Hank's balanced salt solution (HBSS). The tubes were centrifuged at 4°C @ 2000 rpm for 5 minutes in a refrigerated centrifuge. Supernatant was discarded and the cell pellet was further washed two times in HBSS. After the final wash the cells were suspended in sterile RPMI 1640 medium (Sigma, USA) without antibiotics and serum. The cell count was carried out with trypan blue dye to estimate the density of live cells. Approximately 2 x 10^5 cells were seeded in the cell culture plates (Corning, USA) and incubated at 25°C for 24 hours. Genotoxic effects of phorate were tested using these cells. Four concentrations of phorate were selected to accomplish the genotoxic experiments in short term cultured cells. The highest concentration selection was made after treating the cells with 500 µg/ml for 3h and calculating the percentage of viable cells using trypan blue dye. Cells showed >98% viability with 500 µg/ml. Thus, the selected concentrations were 4, 20, 100 and 500 µg/ml of phorate.

Whole blood cultured cells of rohu were treated with different concentrations of phorate and the DNA damage was assessed by alkaline comet assay. Controls were taken for comparisons which were without any amount of pesticide. Four different concentrations of the pesticide was dissolved in RPMI 1640, filtered through 0.45µm syringe filters and applied to 24h whole blood cultured cells in triplicates. Three ml of pesticide per concentration was applied to each culture petriplate (size: 60 mm x 15 mm). Control cells received RPMI 1640 treatment only. Cells were incubated with pesticides for 3 hours at 25°C. After incubation, cells were washed with HBSS and re-incubated further at 25°C in RPMI 1640 medium. Three sets of cells from each treatment group and three sets of controls were immediately processed for alkaline comet assay (0h) to evaluate the amount of DNA damage. Other sets in triplicate sets were processed for DNA repair analysis. Triplicate sets of cells of each control and concentration levels were washed and re-incubated in RPMI 1640 media for two different sampling periods. At 1.5h cells from triplicate sets from both control and each of four different concentration levels were harvested and processed for alkaline comet assay and similar event was carried out at 3h incubation in pesticide free medium to observe the DNA repair coverage at 3h of genotoxicant withdrawal.

To carry out alkaline comet assay, cells were removed from culture plates by cell scraper and centrifuged at 5000 rpm for 10 minutes. Cell pellets collected were suspended in chilled homogenizing buffer of PBS and the rest of the process was carried out according to the protocol of Fairbairn et al. (1995) with a few minor modifications. Comet slides were stained with silver stain for visualization [17]. Two slides per plate were prepared and 100 randomly selected non-overlapping cells were scored for comets. The comets were visually assigned a score on an arbitrary scale of 0–4 (i.e., ranging from 0 – undamaged to 4 - maximum DNA damage) based on perceived comet tail length migration and relative proportion of DNA in the comet tail [2]. Visual scoring was done due to the unavailability of the comet software and according to validations made by authors like Collins (2004) who advocated that visual scoring is also very appropriate in the study of genotoxicity. The mean percent of overall DNA damaged cells was calculated by adding the number of cells scored under comet classes 1, 2, 3 and 4, and was termed as total percentage of damaged cells. The extent of DNA damage score in terms of arbitrary units (AU) for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of that class and summing the overall values {may vary within a range of 0 (all cells undamaged - 0×100) to 400 (all cells damaged at class 4 – 4 X 100)} (Feng et al. 2005.) (Fig.1). Time course curves between different doses of the pesticide and AU were obtained.



(a)

(b)

(c)

(d)

(c)

Figure 1: Representative comet images of nuclei from cells of *Labeo rohita*. a. class 0 (undamaged), b. class 1, c. class 2, d. class 3, e. class 4 (maximum damage).

2.3. Statistics

The mean percentage and standard error of each comet class of triplicate sets of petriplates of each concentration level and control were calculated using Microsoft excel data analysis tool pack 2007. Regression analysis was also performed with Microsoft Excel 2007. Nonparametric Mann-Whitney U test was conducted with SPSS statistical package version 10 in order to find out the existence of significant difference between the comets of control and each pesticide treated groups in comet class 0, 1, 2, 3 and 4 at various sampling hours. Test was also performed to find out significant difference in mean percentage of cells with DNA damage and the DNA damage scores expressed in AU among various groups of treatment at each sampling time. Differences were considered significant at a probability level of $p < 0.05$.

3. RESULTS

Table 1 shows the average percentages of five different classes of comets produced by the cultured blood cells of rohu exposed to phorate. The control group cells scored >98 percent comets in the undamaged category of comets (comet class 0) for all sampling hours. Some cells of the control groups also produced comets of class 1, 2 and 3 at various time periods. However, no comets of class 4 were scored at any point of time. In both 4 and 20 µg/ml concentration levels, the percentage of comets scored against comet class 0 increased with a simultaneous decrease in the average percentage of comets in comet class 3 and 4 from 0h onwards. In 100 µg/ml concentration level the fall in the average percentage of comets in comet class 0 was recorded up to 1.5h followed by an increase at 3h. Likewise, in comet classes 3 and 4, an increase in the average percentages of comets was recorded up to 1.5 h followed by a fall at 3h. In 500 µg/ml concentration level, a continuous increase and decrease in the percentage of comets in comet class 0 and 3 occurred from 0h onwards respectively. However, in comet class 4, an increase in average percentage was observed up to 1.5 h with a further decrease at 3 h.

When the total percentages of cells with damaged DNA and the DNA damage scores in AU of treated groups were compared with controls, treated groups with 100 and 500 µg/ml showed significant differences with their respective controls for all sampling periods whereas 4 µg/ml concentration level of phorate showed significant difference only at 0 h, and 20 µg/ml concentration level showed significant difference at 0 and 1.5 h (Table 1). Further, comparing the data among different treatment groups showed that there exist significant differences among all the four treatment groups at all sampling hours, the only exception was between 4 and 20 µg/ml concentration levels at 3 h sampling.

Figure 3 shows the extent of DNA damage in AU with different sampling time for four different concentrations of phorate. The graph showed a significant time-related increase

in concentration levels of 100 and 500 µg/ml up to 1.5h followed by a drastic and significant fall in AU towards 3h. However, significant decline was also observed in 4 and 20 µg/ml concentration level from 1.5h onwards.

Table: 1. DNA damage measured by alkaline comet assay {frequency of cells in each comet class (%). DNA damaged cells (%) and DNA damage scores in arbitrary units (AU)} in cultured blood cells of *Labeo rohita* following exposure to different doses of phorate for 3 hours and subsequent transfer to normal medium for various time periods.

Hours of exposure	Phorate doses (µg/ml)	Number of cells in each comet class (mean ± SD)					DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)
		0	1	2	3	4		
0	0.00	99.16 ± 0.16	0.83 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.83 ± 0.16[a]	0.83 ± 0.16[a]
	4µg/ml	93.00 ± 0.36*	0.66 ± 0.21	1.66 ± 0.21*	1.00 ± 0.25*	3.66 ± 0.33*	7.00 ± 0.36[b]	21.67 ± 1.08[b]
	20µg/ml	87.83 ± 0.47*	1.83 ± 0.16*	2.66 ± 0.44*	2.66 ± 0.21*	5.00±0.25*	12.16 ± 0.44[c]	35.16 ± 1.66[c]
	100µg/ml	67.16 ± 0.60*	19.00 ± 0.36*	5.83 ± 0.30*	4.33 ± 0.61*	3.66 ± 0.21*	32.83 ± 0.60[d]	58.33 ± 1.62[d]
	500µg/ml	51.33 ± 0.49*	25.66 ± 0.21*	9.00 ± 0.36*	7.33 ± 0.21*	6.66 ± 0.71*	48.67 ± 0.49[e]	92.33 ± 2.61[e]
1.5	0.00	98.16 ± 0.30	0.83 ± 0.16	0.83 ± 0.16	0.16 ± 0.16	0.00 ± 0.00	1.83 ± 0.30[a]	3.00 ± 0.68[a]
	4µg/ml	97.50 ± 0.42	0.66 ± 0.21	0.16 ± 0.16	0.50 ± 0.22	1.16 ± 0.16	2.50 ± 0.42[a]	7.16 ± 1.70[a]
	20µg/ml	95.16 ± 0.30*	0.50 ± 0.22	0.66 ± 0.21	1.50 ± 0.22*	2.16 ± 0.16*	4.83 ± 0.30[b]	15.00 ± 0.96[b]
	100µg/ml	59.50 ± 0.42*	4.16 ± 0.54*	3.00 ± 0.25*	8.50 ± 0.34*	24.83 ± 0.16*	40.50 ± 0.42[c]	135.00 ± 1.65[c]
	500µg/ml	72.16 ± 0.16*	0.83 ± 0.16	2.16 ± 0.30*	5.66 ± 0.49*	19.16 ± 0.54*	27.83 ± 0.16[d]	98.83 ± 1.13[d]
3	0.00	98.16 ± 0.30	1.16 ± 0.16	0.33± 0.21	0.33 ± 0.21	0.00 ± 0.00	1.83 ± 0.30[a]	2.83 ± 0.79[a]
	4µg/ml	98.33 ± 0.21	1.00 ± 0.25	0.66 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	1.66 ± 0.21[a]	2.33 ± 0.33[ab]
	20µg/ml	97.16 ± 0.47	0.33 ± 0.21*	0.50 ± 0.22	0.66 ± 0.21	1.33 ± 0.33*	2.83 ± 0.47[a]	8.67 ± 1.96[ac]
	100µg/ml	94.33 ± 0.33*	0.16 ± 0.16*	0.33 ± 0.21	1.83 ± 0.30*	3.33 ± 0.42*	5.66 ± 0.33[b]	19.66 ± 1.42[d]
	500µg/ml	85.83 ± 0.30*	4.66 ± 0.33*	2.33 ± 0.21*	2.16 ± 0.16*	5.00 ± 0.25*	14.16 ± 0.30[c]	35.83 ± 1.19[e]

* signifies significant difference from respective control (0.00 ppm phorate) at $p < 0.05$ by Mann-Whitney U nonparametric test. Different alphabets in DNA damaged cells (%) within each time period indicate significant difference at $p < 0.05$ by Mann-Whitney U nonparametric test.

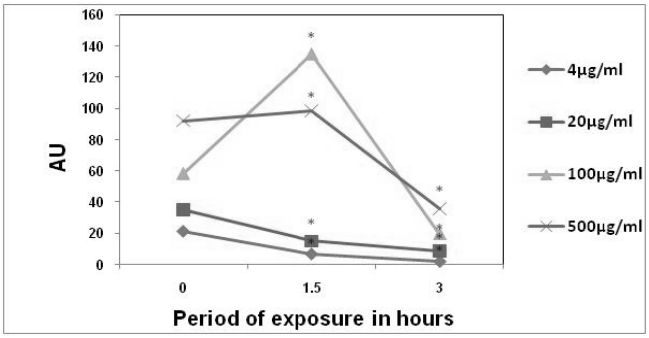


Figure 3: Time course of DNA damage scores in arbitrary units (AU) measured by alkaline comet assay in cultured blood cells of *Labeo rohita* following exposure to different doses of phorate for 3 hours and subsequent transfer to normal medium for various time periods. ***** indicates significant difference from the previous time of exposure within each concentration level at $p<0.05$ by Mann – Whitney U nonparametric test.

4. DISCUSSIONS

Use of living animal models is always a concern for animal loving people and also for others. So an attempt has been made to use short-term cultured cells for the purpose of evaluation of DNA damage and repair studies when living cells get exposed to different types of potential genotoxicants. Phorate, an organophosphate pesticide has always been threat to the integrity of DNA of various animals [18 -19]. Organophosphates class of complexes when undergo through different metabolic itinerary inside a living organisms produces a lot of free radicals which very commonly get in the way with the structural integrity of the biomolecules like protein and nucleic acids [20]. Phorate gets metabolized by both cytochrome P450 dependent monooxygenase system (P450) and the flavin containing monooxygenase (FMO) in living systems particularly in liver cells [21]. These enzymes carry out sulfoxidation of phorate. During this metabolic activity phorate produces lot of electrophiles and nucleophiles as well as some primary metabolites like oxon [20-21]. These electrophiles, nucleophiles and / or other metabolites interfere directly and/or indirectly with DNA molecules and result in production of lesions in DNA strands [21].

Various parameters for genotoxicity measurements have been monitored mainly in permanent cell lines of piscine [22]. An established fish cell line, RTG-2, derived from rainbow trout (*Oncorhynchus mykiss*) has particularly been in use in number of genotoxicity studies [23-24]. Genotoxic potentiality of a pro-mutagen benzo [*a*] pyrene was evaluated in RTG-2 cell line developed from rainbow trout by Castano and Becerill (2004). They used RAPD-PCR DNA fingerprinting technique to distinguish the difference in the banding pattern between the control and the pro-mutagen treated cells. Results obtained from the study clearly indicated the worth, sensitivity, reproducibility and adaptability of in *vitro* system in the genotoxicological studies. Further, the authors suggested that *in vitro* system is useful to assess genotoxic effects, both after acute and chronic exposures and of direct and indirect acting genotoxins. Comet assay has been developed for the detection of DNA strand breaks in cultured cells from aquatic organisms exposed to various genotoxins. Using comet assay, monolayer cultures of rainbow trout hepatocytes exposed to hydrogen peroxide or benzo [a] pyrene as genotoxic model molecules exhibited a significant amount of DNA single strand breaks quantitatively related to the genotoxin concentration [26]. Dose-response increase in DNA strand breakage was recorded in cells of *M.edulis* exposed to both direct-acting (hydrogen peroxide and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[*5H*]-furanone) and indirect acting (benzo[*a*]pyrene, 1-nitropyrene, nitrofurantoin and *N*-nitrosodimethylamine) genotoxins [27]. In the present study, DNA damage induced by phorate in the cultured blood cells of rohu was measured by comet assay. Results indicated that strand breaks were generated by various concentrations of pesticide to different extents as has been shown by increased numbers of class 3 and 4 comets at 0h sampling (Table 1). A number of similar studies have been conducted to detect strand breaks in primary cell cultures of various fish tissues in response to different chemicals. In rainbow trout hepatocytes exposed *in vitro* to B [a] P showed a concentration - dependent increase in DNA strand breaks measured by the DNA alkaline precipitation method [28].

DNA damage revealed by the comet assay in the present study could possibly have originated from single strand breaks, strand breaks during the repair process of DNA strands which involves the formation of gaps in the strands followed by their repair and resealing, DNA adduct formation, DNA–DNA and DNA-protein interacting [29]. They also suggested that the DNA breaks might be taking place due the interaction of pesticides or their metabolites with the DNA molecules. In the present study the pesticide was able to induce genotoxic effects as evident from the results obtained at 0h (Table, 1). Metabolites produced during the time of pesticide metabolism in the culture and their interaction with different chemicals present in culture medium might have contributed in the formation of the DNA lesions in the cultured cells. Interaction of the possible nucleophilic and/or electrophilic nature of metabolites with the DNA molecules could have been able to generate breaks in DNA strands [20].

It has been observed that the pesticides belonging to organophosphate groups are able to produce more genotoxic effects in the cultured cells in comparison with the pesticides belonging to pyrethroids, certain other pesticides, herbicides etc.[30]. However, other pesticides of oganophosphate groups like diichlorvos found to be more genotoxic and cytotoxic to the cultured Chinese hamster ovary cells in comparison to a dinitroaniline herbicide, pendimethalin and a type II pyrethroid pesticide, cypermethrin [30] . In the present study cells treated with lower doses of pesticide on withdrawal of the test compound from medium and further re-incubation in fresh media showed a tendency to repair the damaged DNA over time. It has been observed that this particular pesticide is less accumulative in cells and tissues of the organisms and so do not show its effect for longer period . There was a clear shift in the percentages of comets present in class 3 and 4 towards class 0 from 0h onwards in various doses of the pesticide. Cells exposed to 4, 20 and 100 µg/ml did not show any significant difference in the percentages of cells with damaged DNA and extent of DNA damage at 3h of sampling indicating a total repair of the damaged DNA strands. But the cells of 500 µg/ml of phorate treatment failed to recover the strand breaks by that time as shown by the significant difference in the percentages of cells with damaged DNA and in the extent of DNA damage in AU (Table 1, Fig. 3). However, the higher values for percentages of cells with damaged DNA and extent of DNA damage obtained by the cells exposed to 100 µg/ml can be explained with the probable reason that at higher concentration the chance of cell death might be more due to cytotoxicity whereas at lower doses the

number of cells with damaged DNA might be going through the mechanisms of DNA repair process. Scoring of more comets in class 3 and 4 in 100 µg/ml at 1.5h in indicates the onset of DNA repair process in cells [25]. The quick recovery of the damaged cells in the present investigation might have happened possibly due to the removal the medium which contained the pesticide h along with it all other toxic compounds present in the pesticide itself and the different electrophilic and nucleophilic substances formed during the process of pesticide metabolism by the activation of different detoxification enzyme systems like P450, cytochromes, antioxidant system, [20]. Another aspect of quick recovery of the damaged cells might be due to the presence of erythrocytes in whole blood, which according to some authors plays a detoxifying role by generating the antioxidant system in whole blood cell culture [18-19, 31-32].

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