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ARTICLE

Cytogenotoxic Effect of Pesticides Induces Variability in Micronucleus and Nucleo-Cytoplasmic Abnormalities in Channa punctatus *in vivo*

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ABSTRACT

Our aim was to study the genotoxic, cytotoxic and clastogenic effect of locally used various groups of agro-pesticides in the protection of crops using fresh water fish Channa punctatus as test model to estimate water pollution by micronucleus (MN) assay in vivo. Three different concentrations (MC, MC/2&MC/5) of eight pesticides (Dimethoate, Dichlorovos, chlorpyriphos and Malathion, Methyl parathion, Fenvalerate, Cypermethrin and Carbaryl) at different time periods (5, 10, 15, 20, 25 days) were treated. Peripheral blood samples smears of fish were collected and stained with Giemsa, micronuclei and nucleo-cytoplasmic abnormalities were analyzed under Germany made Leitz microscope. Qualitative analysis shows the rate of concentration, period, nature and mode of action of different agro-pesticides induces varieties of micronuclei and nucleo-cytoplasmic abnormalities even in the same species of fish Channa punctatus in the context of same/different pesticides in different species.

1. Introduction

Human being is the climax victim of each and every environmental pollutant directly/indirectly either naturally born or anthropogenic [1]. Increase in human population and development puts immense stress on protection of crops by pesticides for high yielding to maintain food scarcity throughout the world. Industrial activities and agricultural drainage multi folded pollution, [2-5] discharging waste waters [6,7] to water bodies. These are responsible for multiple effects in the ecosystem and biodiversity in broad range. Carcinogenic and mutagenic compounds effect individual and may be active through following generations. Epizootic neoplasm have been found in a variety of exothermic species, such as shell fish, echinoderms, jawless and bony fish [8].

Fish is excellent model for the study of the clastogenic, mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store water borne pollutants [9-11] similar way to higher vertebrates like human. The main application of fish as a model is to determine the distribution and effects of chemical contaminants in the aquatic environment [12,13] to assess the genotoxicity of water in the field as well as in the laboratory.

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Micronucleus assay was shown to be applicable to fresh water and marine fishes and that gill cells are more sensitive than hematopoietic cells to micronucleus inducing agents. The micronucleus test, developed by [14,15], is an in vivo and in vitro short-time screening method is widely used to detect genotoxic effects. It is one of the simplest, reliable, least expensive and rapid screening system for both clastogenic (chromosome breakage and formation of acentric fragments) and aneugenic (chromosome lagging and effects on spindle) effects [15-21].

The formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes by [22] NAs, including lobbed (LB), blebbed (BL), and notched (NT) nuclei, bi nucleated (BN) cells and many others have been used by several authors as possible indicators of genotoxicity. Several studies have shown that erythrocytes of fish present a high frequency of micronuclei and nuclear abnormalities after exposure to different heavy metals under both field and laboratory conditions [6,12,23].

The detection of MN and NAs in fish helps us to assess the status of water quality as well as the health of a particular species and any potential risk it might have after consumption [24].

The purpose of our study was to evaluate the cytogenotoxic (clastogenic or aneugenic) effects of Dimethoate, Dichlorvos, Chloropyriphos methyl parathion, Malathion, Fenvaleate, cypermethrin, carbaryl are the organophosphorous, pyrethroid and carbamate group of insecticides of different doses individually induces variability in Micronucleus and Nucleo-Cytoplasmic Abnormalitiesin Channa punctatus in vivo using the Micronuclei Test(MNT) [25-28].

2. Material and Methods

Test animal: Specimens of live fish Channa punctatus measuring about 10-12 cm collected from the local ponds and maintained in laboratory aquaria were used for seven days before treatment.

Test Chemical: Dimethoate, Dichlorvos, Chloropyriphos, Methyl Parathion, Malathion, Fenvalerate, Cypermethrin, Carbaryl are the organophosphorous, pyrethroid and carbamate group of insecticides belongs to different trade name and manufacture bought from the local market.

Doses and route of exposure: From among the specimens acclimatized for at least a fortnight in the laboratory aquaria, only strong and active fishes were released into different aquaria containing pesticides correspond to $LC_{50,}$ MC, MC/2 and MC/s doses respectively as per Table 1.

Micronucleus Test: The smear of peripheral blood drawn from the caudal vein with a heparinized syringe, was prepared and well-dried slides were stained in 10% Giemsa solution (Stock solution diluted with Sorensen's buffer at pH 6.8) for 30 min following the method of [14]. Four thousand cells per animals (1000 cells per slide) were scored for micro-nuclei and nuclear anomalies.

3. Results

Erythrocytes of Channa punctatus have a fairly large smooth centrally placed, elliptical nuclei and sizeable cytoplasm. The ratio of nucleus to cytoplasm is about 1:5. The smears of all the treated group of specimens,

Table 1. List of pesticides used in the present study along with their LC50,MC,MC/2 and MC/5 concentrations.

Pesticide(Trade name)	Manufacturer	LC50(in µg/liter)	MC/2(inµg/liter)	MC/2(inµg /liter)	MC/5(inµg/lite)
Dimethoate(Roger-30E)	Rallies India Ltd.,21,D,Sukhadev Marg,Mumbai-400001,India.	100	50	25	10
Dichlorovos(Nuvan)	Hinustan Ciba-Geigy Limited,14. J.Tata Road,Mumbai-400020,India.	500	250	125	50
Chlorpyriphos(Tafaban-20E)	Rallies India Ltd.,21,D,Sukhadev Marg,Mumbai-400001,India	10	5	2.5	1
Methyl Parathion (Metacid-50)	All India Medical Corporation,185, Princess Street,P.B.No.2398, Mumbai,India.	300	150	75	30
Malathion(Mal-Tox)	All India Medical Corporation, 8thRoad, AkhandJyotiBuilding, SantaCruez East Mumbai-400020,India.	250	125	67.5	25
Fenvalerate (Sumicidin)	Rallies India Ltd.,21,D,Sukhadev Marg,Mumbai-400001,India	250	125	67.5	25
Cypermethin(Polytren-20E)	Solar FARMACHEM Ltd. Sorodhi,Valsad,Gujarat.	10	5	2.5	1
Carbaryle (Sevin)	Rallies India Ltd.,21,D,Sukhadev Marg,Mumbai-400001,India	250	125	67.5	25

irrespective of the pesticides concentrations or the period of exposure examined in this study, revealed consistent variations from the above mentioned normal feature of erythrocytes in significantly high frequency than in control using Piscean micronucleus test [13,26,29-39] and thus were not artifactual. In other words, the anomalies did not result due to technical protocols followed in this study but were, in fact, produced due to action of pesticides. The following were the types of nuclear lesions and Nucleocytoplasmic Abnormalities observed in this study as [40] and other workers [41-52].

(1) Micronuclei

Non-refractive cytoplasmic particles with a distinct and characteristics consistent with stain intensity of the nucleus were considered as micronuclei. The size as well as location of such particles in the cytoplasm varied from cell to cell but the shape was almost round or oval in all cells examined. In majority of cells, however, they appeared as minute dot with diameter varying from 1/5to 1/20 of the main nucleus. Furthermore, in many cells they were placed very close to the nucleus and appeared to be connected to the latter a very thin basophilic strand (Figure 1A). Again, each affected cells usually has a single MN but cells with two or more MNs were not completely absent from the preparations (Figure 1B-1C, 2A-2M, 3A-3L, 4A-4G).

Nuclear and Nucleo-cytoplasmic Anomalies

(2) Notched Nuclei (Figure 1D, 5B-5C)

A nucleus with a well-defined slit of uniform width extending to an appreciable depth into the nucleus without no nuclear material and seemed to be demarcated by the nuclear envelope.

(3) Blebbed Nuclei (Figure 1E-2F, 5D-5E):

Nuclei with the small nuclear envelope evagination. The size of the blebs in the majority of the cells with blebbed nuclei was similar to that of the micronuclei. However, the size of different blebs varied, from cell to cell, from a slight protrusion to a slaked structure and round terminus.

(4)Dumb bell shaped(Figure 5F)

(5)Lobedd Nuclei (Figure 1G, 5G-5H)

Nuclei with evaginations larger than blebs was recorded as 'lobed nuclei'. In the majority of the cells, the latter appeared as cross or 'X' Shaped.

(6)Conical nuclei (Figure 1H-1I, 6A)

In many cells nuclei assumed a cone shape due perhaps to the presence of a well-connected micronucleus near them and space in between little lightly stained.

(7)Budding (Figure 5I-5L)

(8)Vacuolated Nuclei (Figure 1J, 6B-6I)

(9)Disintegration (Fig. 1L, 6L)

And ultimately to the disintegration of the nuclei in groups of specimens exposed for longer period to higher concentration (MC) of the pesticides.

(10)Binucleated cells (Fig. 1J, 7A-7F)

Presence of two nuclei within the cell which is indicative of failed cytokinesis. It was found that higher frequency of chromosomal disjunction occurs such binucleated cells than those cells with completed cytokinesis [7].

(11) Broken egg nucleus (Figure 9E-9H)

(12) Retractor Nuclei (Figure 9I-9L)

(13)Cells with condensed chromatin(Fig. 13D-E)

Chromatin aggregation is extensive the nucleus may appear to be fragmenting [8].

(14)Pyknotic cells Fig.12C

Cells characterized by a small shrunken nucleus which contains a high density of nuclear material [7].

(15)Karyorrhectic cells (Fig. 4J, 13J)

Cells with nuclear disintegration and the loss of integrity of the nucleus [7,8].

(16) Karyolytic cells (Fig. 4K-4L, 8K)

Cells in which the nucleus is completely depleted of DNA and is apparent as a ghost like image.

(17) Fused nucleus (Fig. 7G)

(18) Twisted (Fig. 7H)

(19) X shaped with MN (Fig. 7I-7J)

(20) Tear drop like nuclei (Fig. 7K)

(21) Sickle shaped and MN (Fig. 7L)

(22) pin worm (Fig. 8A)

(23) Saucer (Fig. 8B)

(24) Tadpole (Fig. 8C)

(25) kidney (Fig. 8D)

(26) Heart (Fig.8E-8F)

(27) Hooked (Fig. 8H-8I)

(28) Deformed nucleus (Irregular Shaped Nucleus) (Fig. 9A-9D)

(29) Condensed nuclei (Figure 10A-10K)

(30) Terminal nucleus (Figure 10L)

(31) Echinocytic nucleus (Figure 11A-11C)

(32) Swollen Nucleus (Figure 11D)

(33) Elongated (Figure 11E)

(34) Trilobeed (Figure 11F)

(35) Nuclear budding (Figure 11G-11I)

(36) Apoptosis (Figure 4H-4J, 11J-11K)

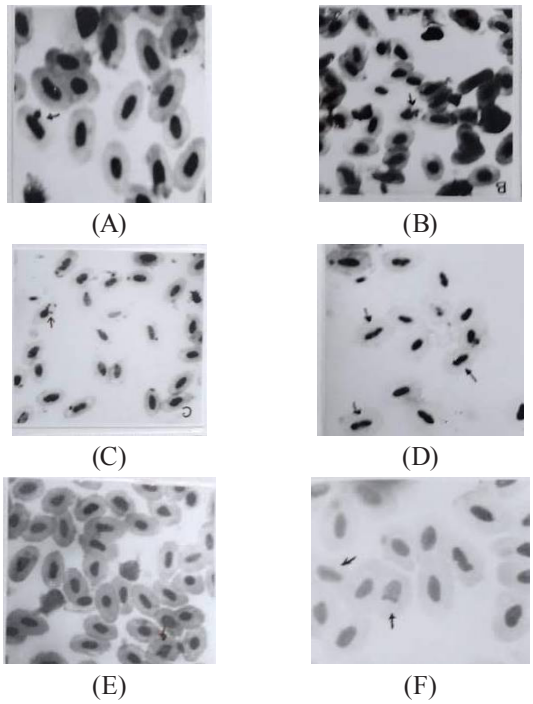
(37) Necrosis (Figure 11L)

(38) Hooked Nucleus (Figure 8G-8H)
(39) Microcyte (Figure 12A, 13B)
(40) Stomatoocyte (Figure 12B)
(41) Discocyte (Figure 12D)
(42) Echinocyte (Figure 12E)
(43) Astrocyte (Figure 12F-12G)
(44) Tailed cytoplasmic process (Figure 12H-12K)
(45) Twin Cell with Cytoplasmic bridge (Figure 12I)
(46) cytoplasmic bud (Figure 12J-12L)
(47) Anisochromiasis (Figure 13A) Cytoplsmic Abnormalities (CA) pigmented periphery and a virtually colourless central region
(48) Fused Cell (Figure 13C)
(49) condensed chromatin lobe (Figure 13D-13F)
(50) enucleated microcyte (Figure 13G)
(51) Spindle Shaped nucleus (Figure 13H)
(52) Large Nucleus (Figure 13I)
(53) Sickle shaped cell vacuolated bud (Figure 13K)
(54) vacuolated cytoplasm (Figure 13L)
(55) Enucleated (EN): without nucleus (Figure 8L)
(56) Nuclear bud (Figure 11G-11I)
(57) Nuclear bridge (Figure 5A)
(58) Cell with damaged Nucleus (Figure 8J)
(59) Anisochromiasia (AN): Cytoplsmic Abnormalities (CA)s pigmented periphery and a virtually colourless central region (Figure 13A)

In vitro evaluation of genotoxicity using the micronucleus assay and nuclear abnormalities in Teleostei, Gobiidae in water sample [48]. *Clarias gariepinus* (Burchell 1822) [53] and native fishes from the Paraná river (Argentina) [54] shows variety of Micronculei and nuclear anomalies. More similiarresulits found out in vitro in fish analyze the incidence of micronucleus and nuclear anomalies in the blood cells of fresh water fish Cirrhinus mrigala treated with Chlorpyriphos [55] Malathion [56] on Channa punctatus, [57] and rats. Induction of micronuclei and erythrocyte alterations in the catfish *Clarias batrachus* by 2,4-dichlorophenoxyacetic acid and butachlor [58]. Induction of micronuclei and nuclear lesions in Channa punctatus following exposure to carbosulfan, glyphosate and atrazine [59]. Nuclear and Cytoplasmic abnormalities in the fish *Catla catla* (Hamilton) exposed to chemicals and ionizing radiation [59]. In vivo and in vitro exposure for the evaluation of the genotoxic effects of lead on the Neotropical water fish *Prochilodus lineatus* [61]. In our study we also observed karyolysis, pyknosis, karyokinesis like Enuclear necrotic cells as [62] and apototic bodies are not exempted.

Morphological changes in normal erythrocytes (discocytes) are dose dependent obtained from blood samples of healthy volunteers are subjected to shape transformation in to stomatocytes and echinocytes treatment with various concentrations of Triton x 100 and Sodium salicylate, respectively [40]. In a sharp contrast to the results obtained by [46] was species differences in micronucleus induction of the clastogenic compounds associated with metabolic profile. [63] find that chromosomes from micronuclei may trigger a chromosomal instability phenotype disaggregating at the mitosis following MN formation. Atrazine effect of toxic blooms on wild fish populations [45] established a correlation between chemical composition of each compound, dose time period and mechanism of action of metabolites in different fish species of different location may cause variety of micronuclei and nuclear Anomalies in fish. The frequencies rates of MN, NAs in addition to Morphologically Alterd Eythrocytes (MAE) may exhibit significant variation depending upon the nature and kind of the toxic agents [2]. [44] said the frequencies in rats [5] supported influence of factors like fish species, class, dose and concentration of the pesticide and exposure time in enhancement of Piscine micronucleus.

Micronucei and nucleocytoplasmic anomalies (Group microphotograph)



(A) (B) (C) (D) (E) (F)

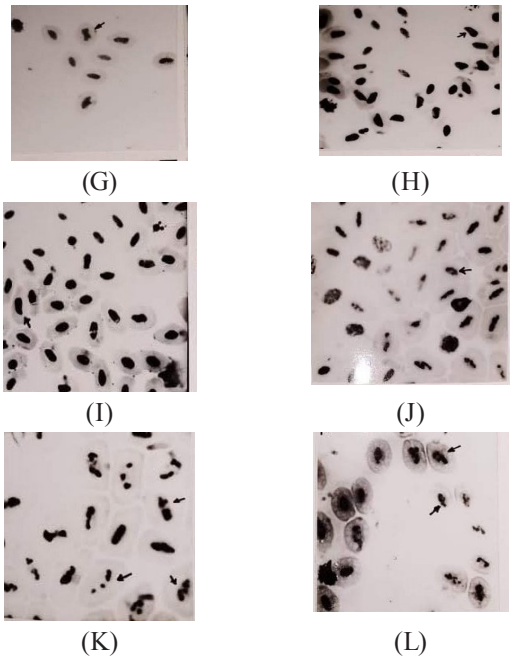


Figure 1. Microphotograph showing erythrocytes with (A) Single micronucleus. (B-C) two and four micronuclei (D) notched nucleus (E-F) blebbed nucleus. (G) lobed nucleus. (H-I) conical nuclei (J) vacuolated nucleui (K) fragmented and disintegrating nucleus. (L) disintegrating nuclei in erythrocytes

Micronclei (MN)

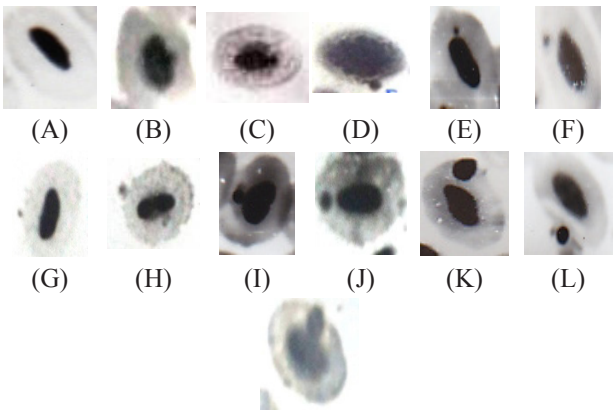
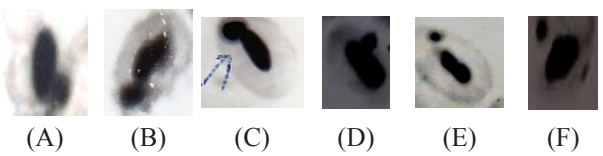


Figure 2. Microphotograph showing erythrocytes with (A) erythrocyte with normal nucleus (B) erythrocyte with normal nucleus with micronucleus bud (C) -(L) Single micronucleus in different shape, size and position,



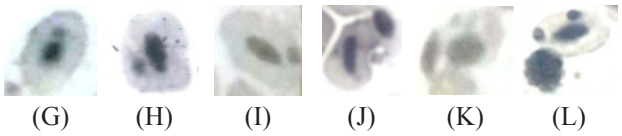


Figure 3. Microphotograph showing erythrocytes with (A) -(I) normal nucleus with micronucleus (J) -(K) Single Notched Nucleus with micronucleus in different shape, size and position, (L) two micronucleus with a condensed nuclear bud

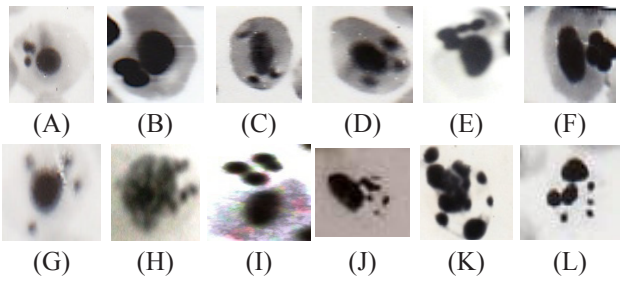


Figure 4. Microphotograph showing erythrocytes with (A)-(B) two micronuclei in different shape, size and position (C)-(D)three micronuclei (E)-(G) four micronucleus in different shape, size and position (H-J) apoptic bodies (J) nuclear fragmentation (karyorrhexis) (K-L) nuclear fading (karyolitic).

Nuclear -Cytoplasmic Anomalies

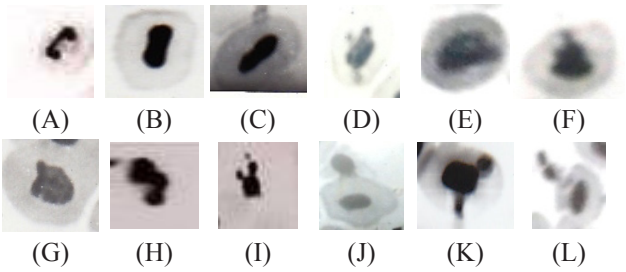


Figure 5. Microphotograph showing erythrocytes with (A) Nuclear Bridge (B)-(C) Notched (D)-(F) Blebbed (G)-(H) Lobed (I)-(L) Budding

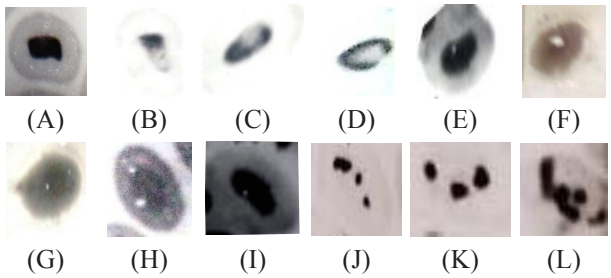


Figure 6. (A) Conical (B)- (I)Vacuolated (J) - (K) Fragmented (L)Disintegrated

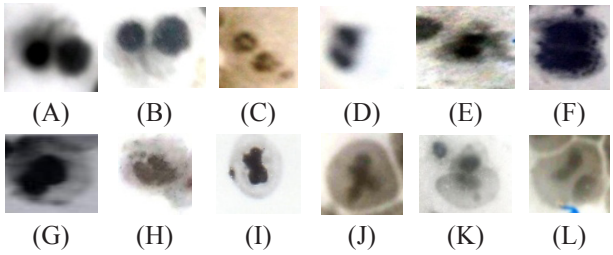


Figure 7. Microphotograph showing erythrocytes with (A)- (E) Binucleated (F) Binucleated with MN (G) Fused nucleus (H) Twisted (I) X shaped and MN (J) X shaped (K) Tear drop like nucluei (L) Sickle shaped

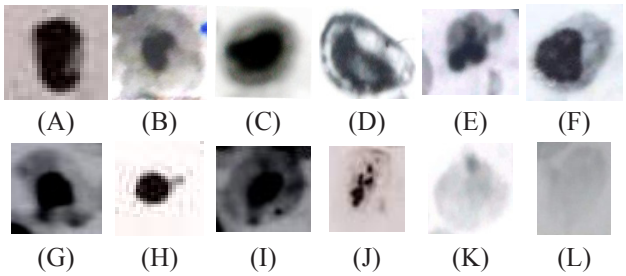


Figure 8. Microphotograph showing erythrocytes with Nucleus (A) pin worm (B) Saucer (C) Tadpole (D) kidney (E)-(F) Heart (G)-(H) Hooked (I) Retracted nuclei with MN (stomata) (J) damaged nucleus with MN (K) Terminal (L) Enucleus

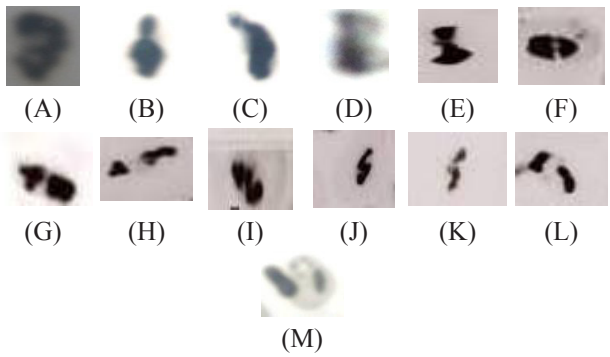


Figure 9. (A)-(D) Deformed nucleus (E)-(H) Broken egg nucleus (I)-(L) Retractor Nuclei

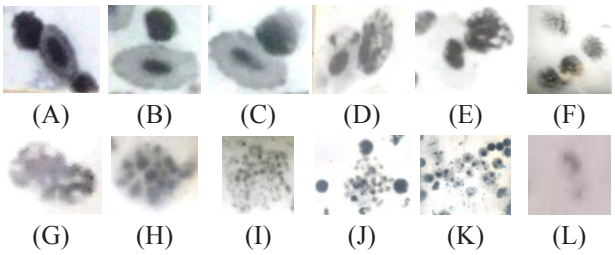


Figure 10. (A) Bilobed Condensed nucleus(B)-(C) lobed condensed nucleus (D)-(E) Bimicronucleated lobed condensed nucleus (F) micronucleated cell with condensed lobe (G) apoptic Nucleus (H)-(K)-condensed chromatin (L) Terminal nucleus

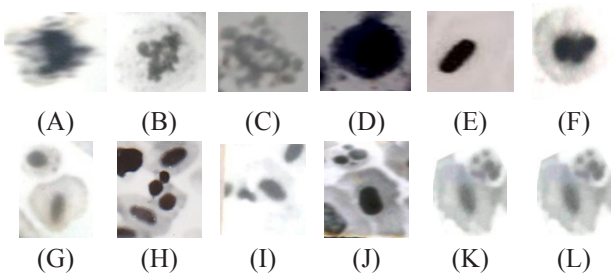


Figure 11. (A)-(C) Echinocytic nucleus (D) Swollen Nucleus (E) Elongated (F) Trilobeed (G)-(I) Nuclear budding (J)-(k) Apoptosi (L)Necrosis

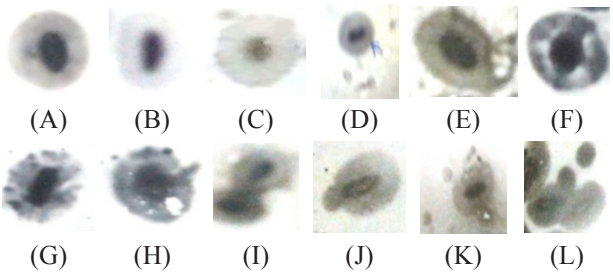


Figure 12. (A) Stomocyte (B) Discocyte (C) pyknotic (D) Microcyte (E) Tailed cytoplasmic process (F)-(G) Astrocyte (H) vacuolated cytoplasm (I) Twin Cell with Cytoplasmic bridge (J) cytoplasmic bud (K) vacuolated Cytoplasmic bud (L) Tear Drop cytoplasmic bud

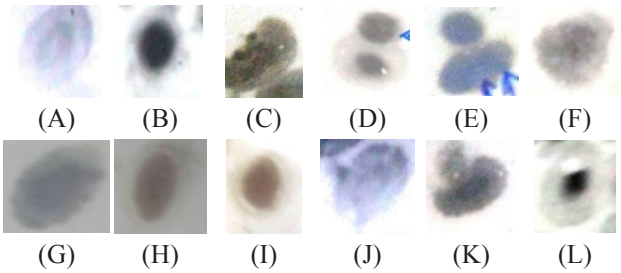


Figure 13. (A) Anisochromiasia (B)Microcyte (C) Fused Cell. (D)-(E) condensed chromatin lobe (F) conical condensed microcyte (G) enucleate microcyte (H) Spindle Shaped nucleus (I) Large Nucleus (J) Karyohexis (K) Sickle shaped cell withvacuolated bud (L) vacuolated cytoplasm.

4. Discussion

Micronuclei are supernumerary nuclei visible by light microscopy in the cytoplasm of hematopoietic or sometimes even in actively dividing cells, called "Howell-Jolly Bodies" in mammals [63], they are formed in dividing cells when acentric chromosome fragment (s) or whole chromosome lags behind during anaphase of clastogenic or aneuploidic events [65-67]. Although the test was originally developed and standardized using rodent bone marrow

cells [15,65,68], it has been shown to work well with peripheral blood [69], Meiotic cells [70], liver cells [71] etc. of rodents, red blood cells of news [72,73] and peripheral blood and kidney cells of fish [26,29,31,34,36-38,74]. Utility of micronucleus test as in situ indicator of biological effects in wild fish, has, however, been doubted by [28,74] for two main reasons. First, occurrence of micronuclei in extremely low frequency in the dividing cells in fish species so far investigated as compared to rodents and second, a lack of a significant correlation between the variations in nuclear morphology and the level of chemical contamination in the sediment or the bile or liver of the specimens of while croaker, *Genyonemus lineatus* [75] investigated. In sharp contrast to the observation of [75], we observed a very consistent and significant in the nuclear morphology and micronuclei in all the groups of specimens of *Channa punctatus* exposed to different pesticides as compared to the controls. We also observed a significant increase in their frequency with the increase in the concentration of the pesticides as well as the period of exposure (vide infra). This clearly suggests that the various kinds of erythrocyte nuclear lesions including micronuclei observed during this study must have originated from a genotoxic event as a result of exposure of the specimens to pesticides. A further support to our contention comes from observations of [13]. While developing suitable genotoxicity assay systems based on aquatic organisms, these authors observed high frequency of micronuclei in the gill epithelia of Carassius sp. (Funa) and *Zacco platypus* (Oikawa) collected from mid-stream of the river Tomio (Nova, Japan) as compared to those collected from upstream of the same river. They also observed structural chromosome aberrations as well as micronuclei in the cells of embryos *Rhodeus ocellatus* (Rose bitterling) grown in water containing trichloroethylene and evaluating Micronucleus Test's sensitivity in freshwater fish check changing of genome, [50,76]. Variability in micronucleus induction with different mutagens applied to several species of fish. But here we differ with [77] using a single species with eight different agro pesticides obtained more variable Micronucleus (MN), Nuclear anomalies (NA) and Nucleo-Cytoplasmic Anomalies (NCA) [52] which is highly significant one.

Emergence of micronclei and their effects on the fate of cells under replication stress was studied by [78] is further supported by DNA Breaks and Chromosome Pulverization from Errors in Mitosis by [79]. Many more deep study and co-relation regarding mechanism of action of mutagens, carcinogens and clastogens is essential among the workers for origin of micronucleus and nuclear anomalies in future. Hence said chromosome aberrations and micronuclei tests may prove to be powerful sensitive assays for detecting genotoxins in aquatic environment.

Suggests that induction of cell death, ghost cells, cells with membrane damage and binucleted cell by cytotoxic and genotoxic effects of the Inula viscose leaf extracts on Allium cepa [80], as a Consequence of global warming [51] found frequency of erythroblasts (Ebs), erythrocytic nuclear abnormalities (ENA) and erthrocyttic cellular abnormalities (ECA) were increased in response to thermal stress in common carp Cyprinio carpio. Comparing Cellular alterations in fish exposed to ionisising radiations and pesticides, [60] in order to identified micronuclei Assay as biomarker of radiation also renamed as the Erythrocytic micronucleus Cytome assay (ECMNA) as it encompasses variety of biomarkers that may find application in genotoxicity. Micronuclei and Nuclear Abnormalities increases during increased the days of exposure of sub lethal Karanjin obtained from seeds of plant Pongamiaa pinnata in Fish Cyprinio carpio [81].

5. Conclusions

Our study suggests, genotoxic pesticides with variation in structural organization, functional group, different mode of action and mechanism of function in same tissue of same species of fresh water live fish *Channa punctatus* (Bloch) induces variety of Micronuclei, Nuclear Nucleo Cytoplasmic Abnormalities (NCA) with respect to time period and concentration dependent which differs from [76] in different species. In the subsequent trophic level at the end point of food chain the most sufferer will be the human being through biomagnifications of mutagenic, carcinogenic, clastogenic and teratogenic pollutants [8] Hence rapid and urgent alternatives are necessary for both agricultural practices, industrial development [82] as well as the survival of flora and fauna including human civilization in a healthy environment.

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