

Histopathological, Hematological and Genotoxic Study on *Channa punctata* (Bloch 1793) Treated with a Sub-Lethal Dose of Organophosphate Pesticide Malathion

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ABSTRACT

Application of pesticides could represent a contaminant source for the aquatic environment and thus affect aquatic fauna. Fish are often used as indicators of pesticide water contamination. Therefore, the present study deals with the effect of Malathion, an organophosphate insecticide, on the histological, hematological (total leukocyte count), and genotoxic study on a freshwater teleost *Channa punctata*. In the present study, the effect of a sub-lethal concentration of 1.3 ml/L of Malathion (1/5th of LC₅₀) for 24, 48, 72, and 96 hrs on hematological changes (total leukocyte count), Histopathological changes in gill, liver and kidney and genotoxic effect through micronucleus test of *C. punctata* was studied. The WBC count increased during the study's exposure period. Structural variations like epithelial lifting, fusion and curling of secondary lamellae, hyperplasia in secondary lamellae, and aneurysms were observed in fish gills; distended sinusoids, necrosis and vacuolation in the fish liver were observed. Moreover, necrosis, vacuole formation, degeneration of tubular epithelial cells, degeneration of interstitium, and necrosis in hematopoietic tissue were observed in the kidney of *C. punctata*. The mean frequency of micronuclei induction increased with the exposure time, which was the highest in 96 hrs of treatment.

Key words: Insecticide, Fish, Hematology, Histology, Micronucleus

INTRODUCTION

India is primarily agro-based with more than 60-70% of its population dependent on agriculture. However, due to pest infestation, 30% of its agricultural produce is lost. Therefore, there is a growing need for agrochemicals like insecticides, fungicides, pesticides, and herbicides to safeguard crops in one way or another. About 3% of the world's herbicides are utilized in India, rising by 2-5% annually (Bhadbhade et al. 2002). India currently ranks twelfth in the world and is the second-largest pesticide maker in Asia after China (Mathur 1999). In India, 76% of the pesticide used is insecticide, against 44% globally. The primary use of pesticides in India is for cotton crops (45%), followed by paddy and wheat (Srivastava et al. 2016). Synthetic pesticides such as organophosphate insecticides have been used since the 1960s, carbamates in the 1970s, pyrethroids in

the 1980s, and herbicides and fungicides in the 1970s -1980s as great contributors in controlling pest and agricultural output (Anonymous 2001).

Among these pesticides, organophosphorus compounds are commonly used as insecticides. Malathion (O, O- dimethyl phosphodithioate of diethyl mercaptosuccinate), a commonly used organophosphate, is applied for mosquito control and to combat agricultural pests. Malathion is not considered a persistent pesticide. It is used for both agricultural and non-agricultural purposes. Once Malathion is introduced into the environment, usually from spraying on crops or in vast urban or residential areas, droplets of Malathion in the air fall on soil, plants, water or man-made surfaces. While most of the Malathion will remain in the regions where it is used, some may be carried by rain, fog, or wind to other locations. Malathion dissipates rapidly in water owing to the water and bacteria present there. It

decomposes in the atmosphere by reacting with other compounds from sunlight to generate the more hazardous substance known as Malaoxon (Larramendy and Soloneski 2015). Once Malathion is introduced into the environment, it may seriously intimidate aquatic organisms and cause severe metabolic disturbances in non-target species like fish and freshwater mussels. The acute toxicity of Malathion and its active primary metabolic product Malaoxon, results from the impediment of acetylcholine degradation at the neuromuscular junction through irreversible inhibition of acetylcholinesterase (Pugazhvendan et al. 2009).

It is well known that applying pesticides could represent a contaminant source for the aquatic environment and thus affect aquatic fauna particularly fish. Fishes have the potential to respond to low concentrations of pesticides and they show physiological and behavioural changes hence they are often used as indicators of pesticide contamination in water. Thus, monitoring sentinel fish species is widely used to assess the degree of toxicant accumulation and their effects on health status. In teleost fish, the tissues most frequently used in eco-toxicological and pathological investigations are the gills, liver, kidney, and muscles.

Channa punctata (Bloch 1793), a Perciformes commonly known as spotted snakehead (Goroi in Assam, India), is an important fish found preferably in stagnant muddy streams, ponds, brackish water, beels, etc. It is available throughout the season, has a wide distribution, easily acclimatized to laboratory conditions, and has high commercial value. All these characteristics make this species an excellent test specimen for toxicity studies (Stoyanova et al. 2015).

MATERIAL AND METHODS

Collection of the specimen

Healthy specimens of *Channa punctata* (Bloch) of average length (12 ± 2 cm) and weight (25 ± 3 g) (Family: Channidae; Order: Perciformes) were collected from the local fish market, of Maligaon, Guwahati (Assam). Fishes were brought to the Cell and Molecular Biology Laboratory of the Zoology Department, Gauhati University, and were treated with 0.1% KMnO_4 solution for 2 minutes to avoid dermal infection. They were then acclimatized under

laboratory conditions for 15 days in a glass aquarium (75 L capacity) maintained at pH - 7.23 ± 0.05 , Temperature - $26.30 \pm 0.57^\circ\text{C}$, Photoperiod - 12:12 hrs light and dark regime. Fishes were fed daily with nursery fish feed (Manufactured by CPF India Pvt. Ltd.) during acclimatization periods. Each day, 70% of the water volume was renewed to maintain water quality and to remove the excretory waste and food debris.

Procurement of the test chemical

For the present study, commercial grade Malathion (50% EC, Excel Fertilizers Ltd. India) was procured from the local supplier (Indo Seeds Farm of Machkhowa, Guwahati, Assam).

Experimental design

Based on the literature studies, the LC_{50} value for 96 hrs of Malathion for *C. punctata* was recorded as 6.61 ppm (Pandey et al. 2005). Acclimatized fish were divided into two groups: control and experimental groups. Malathion was not added in the control group of fishes while the experimental groups were exposed to a sub-lethal concentration of Malathion (1.3 ml/L). The experiments were conducted in triplicate with 10 fish each. The study was conducted to determine the toxicity in sub-lethal concentrations of the toxicant for 24, 48, 72, and 96 hrs. Feeding was stopped 24 hrs before the initiation of the experiment and the fish were not fed during the entire experimental period. Fishes from the control and experimental groups were sacrificed at 24, 48, 72, and 96 hrs interval. Blood and tissue samples were collected from the sacrificed fish and processed accordingly for histological, hematological, and genotoxic analysis.

Histopathological analysis

For histology, gill, liver, and kidney were dissected from the fishes and then pre-washed in fish saline (64% NaCl) before their fixation in freshly prepared Carnoy's fixative for 3-4 hrs (Hazarika et al. 2015). After fixation, the tissues were subjected to a dehydration process by using ascending grades of alcohol, cleared in xylene, and embedded in paraffin, and paraffin blocks were prepared. The blocks were then trimmed and sectioned under microtome at 5-micron thickness. The sections were then stretched

in a hot water bath, cleared in xylene, and stained using Bancroft's double staining procedure (using Haematoxyline and Eosin stain). After staining, the stained slides were mounted with DPX, and the prepared slides were observed under a compound microscope (Leica 4x-1000 DM 750).

Hematological analysis

For the hematological study, a total leukocyte count was performed. Blood was taken directly by cardiac puncture with the help of a heparinized needle using EDTA as an anticoagulant. The amount of blood is dragged until the 0.5 mark; the blood is then diluted with Turk's fluid up to 11 marks using the pipette. The contents of the pipette were then mixed for a few minutes. The first two drops of diluted blood were then dispelled from the pipette. The Neubauer's chamber was then loaded with the pipette contents by holding the pipette at an angle of 54°. An appropriate drop is allowed to run under the coverslip by capillary action. The corpuscles were allowed to settle down for 2-3 minutes, and then the number of WBCs present in the four large square areas was counted under the microscope at 40 × 10X. The white cells were recognized by their refractive appearance and the slight colour given to them by the Gentian violet dye in the diluting fluid. The WBC of the controlled group was also counted under a microscope.

Genotoxicity test

For the genotoxic study, the micronuclei test was conducted. Blood samples were smeared on clean, grease-free glass slides. Slides were then fixed in methanol for 10 minutes, left to dry at room temperature, and finally stained with 6% Giemsa stain in Phosphate Buffer Saline (pH 6.9) for 20 minutes. After dehydration through graded alcohol and clearing in xylene, slides were mounted using DPX (Nwani and Echi 2013). The slides were then observed under the Leica microscope (Leica 4x-1000 DM 750). Micronuclei were counted per 1000 cells, and frequency was measured.

Statistical analysis

Data were represented as mean ± standard deviation (SD). Statistical analyses were done using SPSS Software (Version 16), and all the treatment means

were tested using One-Way ANOVA followed by Tukey's test.

RESULTS AND DISCUSSION

Histopathological alterations

After exposure to a sub-lethal dose (1.3 ml/L) of Malathion for 24, 48, 72, and 96 hrs, extensive damages were observed in the gill, hepatic and renal tissues of *Channa punctata* fish. No histological structure changes were observed in the control groups. The study found that the tissue deterioration in the Malathion-exposed fishes increased progressively with increase in exposure periods.

Gills

The control group of fishes showed a typical structure of gills with proper secondary lamellae (SL) and primary lamellae (PL) (Figs. 1a-b). During 0-24 hrs and 24-48 hrs of exposure to Malathion, epithelial lifting of secondary lamellae and hyperplasia in secondary lamellae were observed (Figs. 1c-d). During 48-72 hrs of exposure, epithelial lifting (EL), fusion of secondary lamellae, hyperplasia of secondary lamellae, curling of the secondary lamellae, and aneurysms, which represent the severe disturbances in blood vessels were observed (Figs. 1e-f). During 72-96 hrs of exposure, epithelial lifting (EL), aneurysms, hyperplasia of secondary lamellae, and degeneration of epithelial cells were observed (Figs. 1g-h).

Kidneys

Histological sections of kidney tissue of the control group of fish showed typical structure of renal tubules, glomerulus, and Bowman's capsule (Fig. 2a). During 0-24 hrs of exposure of Malathion, only necrosis was observed in the kidney (Fig. 2b). During 24-48 hrs of exposure, necrosis, degeneration of interstitium, degeneration of tubular epithelial cells was observed (Fig. 2c). During 48-72 hrs of exposure also necrosis was observed but comparatively more than that seen at 24 and 48 hrs exposure. Moreover, degeneration of interstitium, degeneration of tubular epithelial cells, and necrosis in hematopoietic tissue was also observed (Fig. 2d). During 72-96 hrs of exposure, extensive necrosis, vacuole formation, degeneration of tubular epithelial cells, were

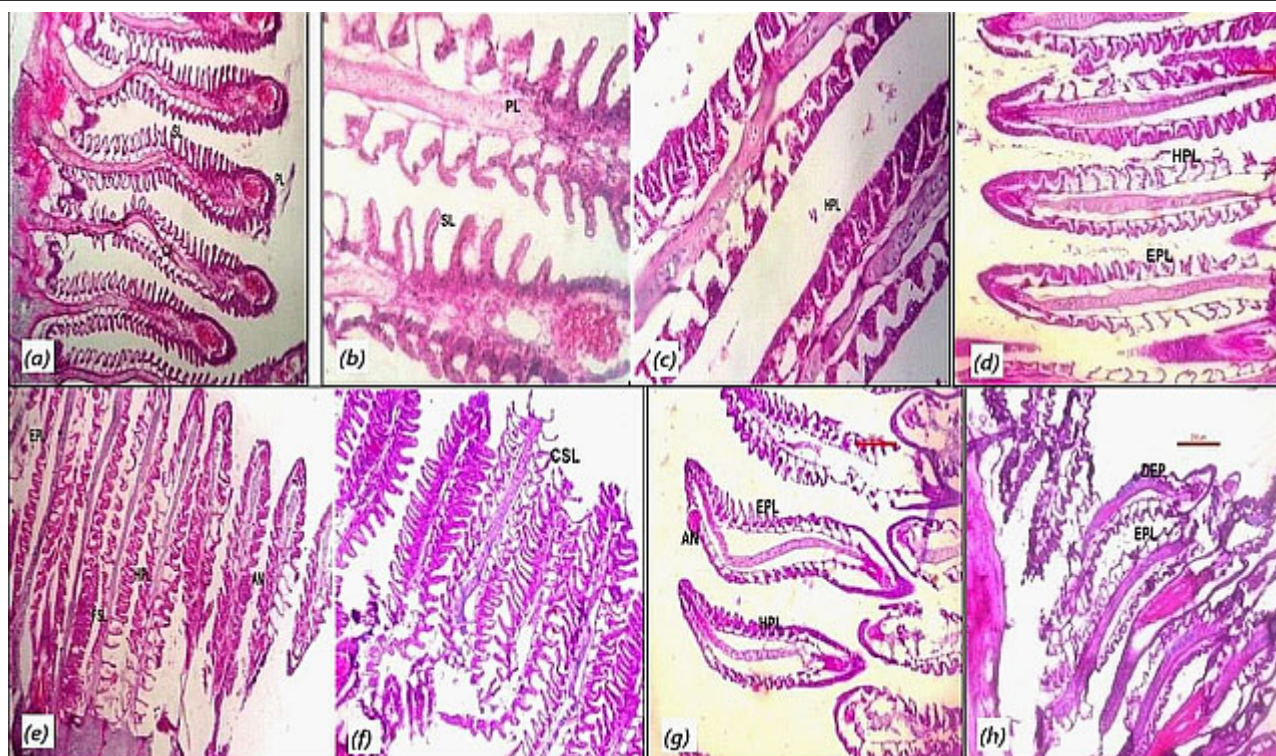


Figure 1. Histopathological alterations in the gills of 1.3 ml/L of Malathion exposed *Channa punctata* at different time intervals compared to control (unexposed) at 100x and 400x: (a-h) Histological section of the gill. (a & b) control showing secondary lamellae (SL) and primary lamellae (PL); (c) After 24 hrs exposure ($40\times 10x$), showing hyperplasia (HPL) in secondary lamellae; (d) After 48 hrs of exposure ($10\times 10x$), showing epithelial lifting (EL) of secondary lamellae; (e-f) After 72 hrs of exposure ($10\times 10x$), showing fusion of secondary lamellae (FSL), aneurysm (AN) in secondary lamellae and curling of secondary lamellae (CSL); (g-h) After 96 hrs of exposure showing degeneration of epithelial cells (DEP) at $10\times 10x$ resolution

observed. Extensive degeneration of interstitium and tissue damage was observed (Fig. 2e).

Liver

The unexposed group of fish showed a typical structure of hepatic cells with bile duct and sinusoids (Fig. 3a). During 0-24 hrs of exposure to Malathion, necrosis and formation of vacuoles were observed (Fig. 3b). During 24-48 hrs of exposure, distended sinusoids, necrosis, and vacuolation were observed (Fig. 3c). During 48-72 hrs of exposure, degeneration of the hepatic cells, distended sinusoids were observed and also necrosis and vacuolation were more during this period compared to 24 and 48 hrs (Fig. 3d). During 72-96 hrs of exposure also necrosis and vacuoles were observed which were comparatively more than the other periods of exposure. Sinusoids were distended, hepatocytes

were degenerated, and an irregular structure of hepatocytes was observed (Fig. 3e).

Hematological changes (total leukocyte count)

The total count of white blood cells (TLC) results revealed that the blood of the control fish showed a mean value of $1.44 \times 10^3 \text{ mm}^{-3}$. The fishes exposed to sub-lethal concentrations of Malathion showed mean values of WBC as $1.72 \times 10^3 \text{ mm}^{-3}$, $1.96 \times 10^3 \text{ mm}^{-3}$, $2.99 \times 10^3 \text{ mm}^{-3}$, and $3.71 \times 10^3 \text{ mm}^{-3}$ for 24, 48, 72, and 96 hrs of exposure, respectively. The values of the WBC count increased with exposure period (Table 1).

Genotoxicity effect

In the micronuclei test, it was observed that the control group of fishes had normal blood cells viewing erythrocytes oval to elliptical with a

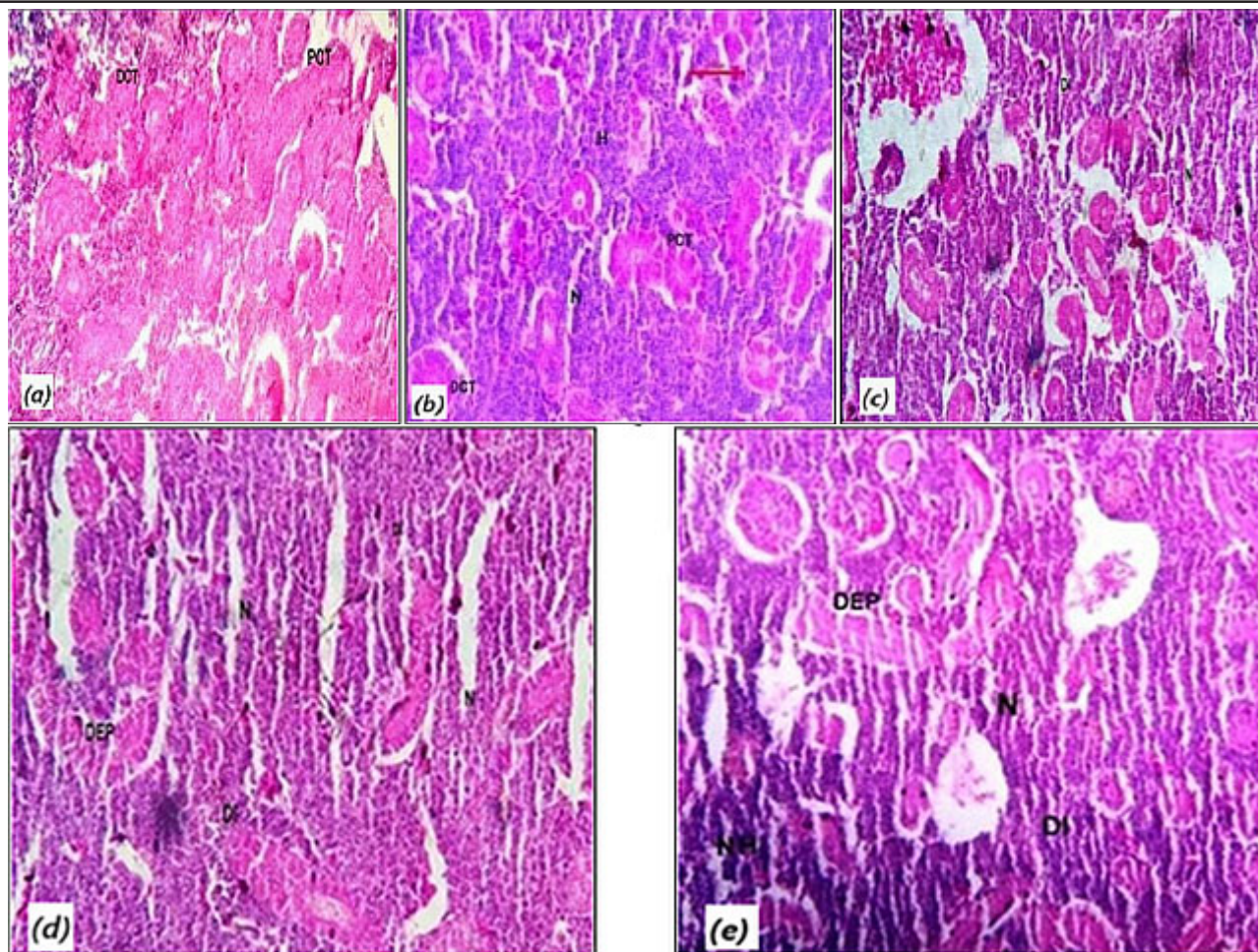


Figure 2. Histopathological alterations in the renal tissue of 1.3 ml/L of Malathion exposed *Channa punctata* at different hours of intervals compared to control (unexposed) at 400x: (a-e) Histological section of the kidney. (a) Control showing proximal convoluted tubule (PCT) and distal convoluted tubule (DCT); (b-e) After 24, 48, 72, and 96 hrs of exposure, respectively, showing necrosis (N), degeneration of interstitium (DI), degeneration of tubular epithelial cells (DEP), and necrosis in haematopoietic tissue (NH)

centrally located oval-elliptical nucleus. Few micronuclei were found (Fig. 4a). While in the Malathion-treated fishes, micronuclei were induced (Figs. 4b-c), and the mean frequency of micronuclei

induction was progressively increased with the increase of the exposure periods with the highest in 96 hrs of treatment (Table 2).

Table 1. Total count of WBC in the control and Malathion treated (1.3 ml/L) *Channa punctata*

Exposure duration (hrs)	WBCs ($\times 10^3 \text{ mm}^{-3}$) (Mean \pm SE)
0 (Control)	1.44 \pm 0.64
24	1.72 \pm 0.34
48	1.96 \pm 0.48
72	2.99 \pm 0.19
96	3.71 \pm 0.10

Table 2. Micronuclei induction in erythrocytes of *Channa punctata* exposed to sub-lethal concentration of Malathion (1.3 ml/L) for a period of 24, 48, 72, and 96 hrs

Time (hrs)	Mean MNRBCs / 1000 RBCs \pm SE	
	Control group	Exposed group
24	0.13 \pm 0.032	0.4 \pm 0.057*
48	0.16 \pm 0.032	0.7 \pm 0.057*
72	0.16 \pm 0.032	1.3 \pm 0.086*
96	0.2 \pm 0.057	1.7 \pm 0.032*

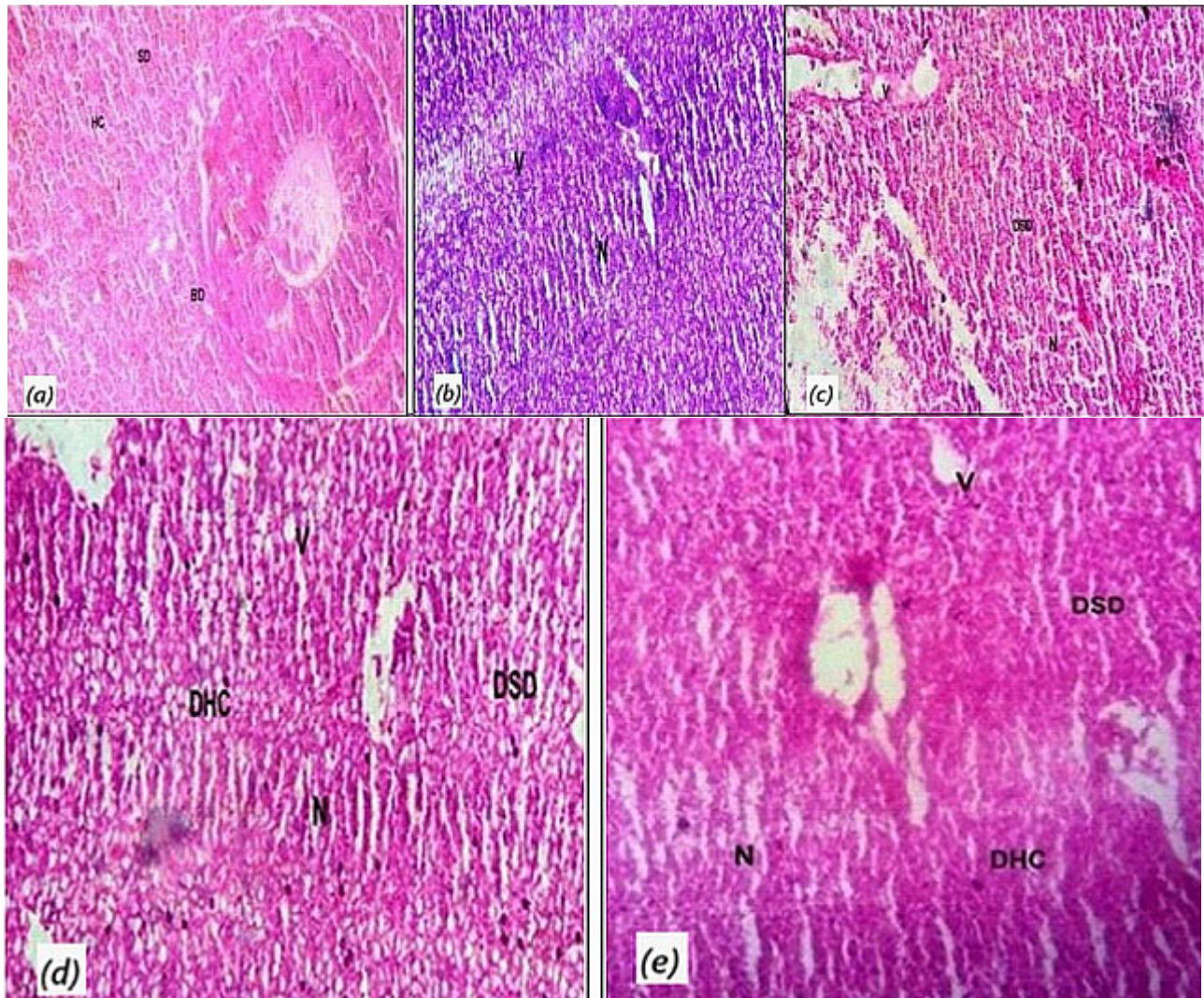


Figure 3. Histopathological alterations in the hepatic tissue of 1.3ml/L of Malathion exposed *Channa punctata* at different time intervals compared to control (unexposed) at 400x: (a-e) Histological section of the liver. (a) Hepatic cells (HC), Bile duct (BD), and sinusoids (SD); (b-e) After 24, 48, 72 and 96 hrs of exposure, respectively, showing necrosis (N) and formation of vacuoles (V), (distended sinusoids (DSD) and degeneration of hepatic cells (DHC)

The gill of fish, when exposed to Malathion, caused several pathological changes, which include epithelial lifting (EL), fusion of secondary lamellae, degeneration of secondary lamellae, hyperplasia of secondary gill lamellae, degenerated secondary lamellae, curling of the secondary lamellae, and aneurysms were also observed after 72 and 96 hrs of Malathion exposure. Similar results were reported by Velmurugan et al. (2009), who observed hyperplasia, oedema, epithelial lifting, and curling of secondary lamellae in the gill of *Cirrhinus mrigala* when exposed to Dichorvos. Hyperplasia, fusion of the secondary lamellae, and epithelial lifting were

considered to be the first degree of gill lesion (Devi and Mishra 2013). Pesticides increase blood vessel pressure, leading to an artery's enlargement or a bulge. This condition is called an aneurysm, the most severe disturbance in the blood vessels that causes changes in the circulatory system (Stoyanova et al. 2015). The histological changes in the gill structure could be a response of the fish organism to toxicant ingestion or an adaptive response of the fish to prevent the entry of the toxicants through the gill surface. Hyperplasia in gill lamellae increases the distance between the external environment and blood; hence, it could be a defence mechanism that

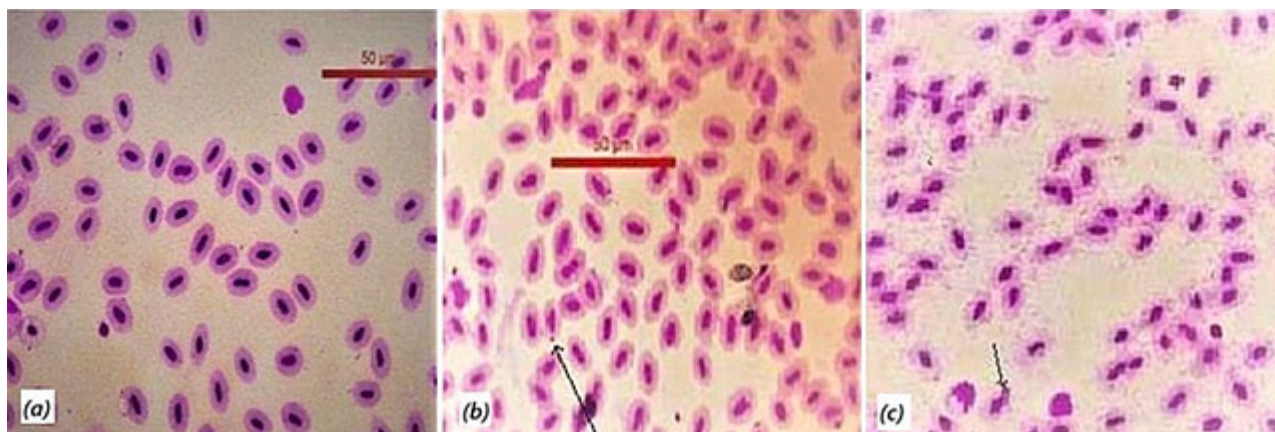


Figure 4. Micronuclei formation in the Malathion treated fish *Channa punctata*: (a) Normal blood cells of control [40 ×10x] showing erythrocytes oval to elliptical in shape with centrally located oval-elliptical nucleus; (b-c) Micronuclei formation in the erythrocytes after 72 and 96 hrs of exposure, respectively

prevents entering different contaminants.

The histopathological changes observed in the kidney of *C. Punctata* when exposed to Malathion were supported by the findings of Sulodia et al. (2014) and Prashanth (2011), who also reported hypertrophy of the tubular cells, vacuolation, perforation of kidney tubules, necrosis in the kidney of *Cirrhinus mrigala* when exposed to Cypermethrin. Such extensive damage in the renal tissues of *C. punctata* exposed to Malathion during the present study illustrates the toxic properties of the sub-lethal concentration of this pesticide.

The histopathological changes observed in the liver of *C. punctata* when exposed to Malathion include degeneration of hepatic cells, vacuolation, necrosis, distended sinusoids, irregular structure of hepatocytes, etc. The damage to the hepatocytes may be due to a lack of sufficient blood supply. Vacuolation of hepatocytes may be due to metabolic damage related to the exposure of Malathion. Butchiram et al. (2009) reported vacuolar degeneration, necrosis, degeneration in the cytoplasm in hepatocytes, and rupture of blood vessels in the liver of *C. punctata*, when exposed to Alachlor, a herbicide. Similar findings were recorded by Deepasree and Nair (2015) in the liver of *C. punctata* when treated with Fytran. The liver has a significant role in detoxification and biotransformation; therefore, due to its function, position, and blood supply, it is the organ most affected by water pollutants. Though it can degrade the contaminated compounds, due to its regulating

mechanisms, it can be inundated with increased concentrations of these compounds and could subsequently result in structural damage (Bruslé and Anadon 1996).

White blood cells are essential to fish defence mechanisms. In the present investigation, the total leukocyte count showed a different pattern change due to Malathion exposure than the control one. The values of WBC count increased with respect to the exposure period. This may be due to immune system stimulation, which causes lymphocyte increase due to tissue damage. Similar results were observed by Parveen and Shadab (2011) in the same fish (*C. punctata*) when exposed to the same pesticide.

The present investigation shows that Malathion induced micronuclei in erythrocytes of the fish *C. punctata*. The mean frequency of micronuclei induction was progressively increased with the increase of the exposure periods, with the highest in 96 hrs of treatment. Parveen and Shadab (2011) found that micronuclei were induced in Malathion treated *C. punctata*, and there was a progressive increase in the percentage of micronuclei with the intensity of exposure. Thus, the technical-grade Malathion has the potential to induce genetic damage.

CONCLUSION

The present investigation shows that Malathion induced several histopathological changes in the gill, liver, and kidney of the fish *Channa punctata*. The toxic effect of this chemical damages the tissues,

which may stimulate the immune system and cause an increase in lymphocytes, which is why the WBC count increases in the fish exposed to Malathion. Moreover, the progressive increase in the mean frequency of micronuclei induction with the increase in exposure periods revealed the clastogenic properties of the pesticide. It showed how fast this chemical can cause genetic damage. Thus, the study revealed that Malathion is a highly toxic chemical that may cause severe damage to aquatic organisms at a very low concentration. Malathion's effects suggested a serious concern about its potential danger to aquatic organisms, especially fish, and indirectly to human beings.

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