Histopathological Effects of Roundup, a Glyphosate Herbicide, on Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT The acute toxicity of Roundup, a glyphosate herbicide, to Nile tilapia, *Oreochromis niloticus*, was investigated with emphasis on histopathological effects. The values of 24-h, 48-h, 72-h and 96-h LC_{50} for young tilapia were 17.5, 17.1, 16.9 and 16.8 ppm, respectively, and those for adult tilapia were 46.9, 44.4, 40.0 and 36.8 ppm, respectively. They indicated that adult fish were more tolerant to Roundup than the much smaller young fish. Roundup concentration corresponding to the 96-h LC_{50} value for adult tilapia was used to study the effects of Roundup exposure in inducing histopathological changes of gills, liver and kidneys. In the gills, filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting and aneurysm were observed. In the liver, there was vacuolation of hepatocytes and nuclear pyknosis. Kidney lesions consisted of dilation of Bowman's space and accumulation of hyaline droplets in the tubular epithelial cells. These changes occurred predominantly in the 96-h exposure.

KEYWORDS: acute toxicity test, histopathology, *Oreochromis niloticus*, Roundup.

INTRODUCTION

The application of environmental toxicology studies on non-mammalian vertebrates is rapidly expanding, and, for aquatic systems, fish have become indicators for the evaluation of the effects of noxious compounds. The indiscriminate use of herbicides, careless handling, accidental spillage, or discharge of untreated effluents into natural waterways have harmful effects on the fish population and other forms of aquatic life and may contribute long-term effects in the environment.

Roundup, the active ingredient which is the 48% acid equivalent of the isopropylamine salt of glyphosate (N-phosphonomethyl glycine), is used as a non-selective herbicide and for aquatic weed control in ponds, lakes and canals. It is perhaps the most important herbicide ever developed.[1] It has been increasingly used in recent years, since it is biodegradable and therefore persists in the environment for only a short time. Because of its low persistence, repeated applications of this herbicide are practiced for the control of weeds in agricultural fields and, thereby, large quantities find their way into water bodies. However, only a few reports have described its effects on freshwater fish.[2-3] The Nile tilapia (*Oreochromis niloticus*) is one of the commercially most important freshwater fish species of Thailand. Nile tilapia are grown in ponds situated near agricultural areas where Roundup is commonly used. Due to this, investigation of its toxicity is very important. Static 96-h LC_{50} bioassays have become the routine in the determination of toxicity of effluents and other chemical substances to various organisms for reasons of consistency, convenience, and control of results.[4]

The 96-h LC_{50} values for glyphosate range from 97 mg/L to 140 mg/L. The operational formulations of the herbicide are more toxic than the technical grade chemical with the 96-h LC_{50} values for Roundup being in the range of 2 mg/L to 50 mg/L.[1] In this investigation, the results were given in terms of the herbicide's commercial formulation and not in terms of the active ingredient of the herbicide used, because only the commercial formulation is used by farmers for agricultural purposes. Thus, the objective of this study was to investigate the acute toxic effects of Roundup on Nile tilapia (*O. niloticus*) with emphasis on the histopathological changes in the gills, liver and kidneys.

Materials and Methods

Animals

Nile tilapia, (*O. niloticus*), used for acute toxicity tests in young fish were 1.69 ± 0.31 g in body weight and 4.50 ± 0.18 cm in length. Those used for acute toxicity tests in adult fish were 16.87 ± 3.87 g in body weight and 9.60 ± 0.91 cm in length. They were purchased from a commercial hatchery in Bangkok, Thailand. Acclimatization to laboratory conditions for 7 days was done using dechlorinated tap water having the following physicochemical characteristics: temperature = 26.0 ± 2.0 °C, pH = 6.8 - 7.3, total hardness = 50 - 70 mg/L (as $CaCO_3$), alkalinity = 62 - 65 mg/L, and conductivity = 185 - 210 μmhos/cm. Chlorine residual and ammonia were below detection limits. These parameters were measured according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater.[4] Fish were fed twice a day with 38 %-protein commercial fish food. The quantity of food was 2 % of initial body weight per day. The commercial formulation of glyphosate (Roundup, the active ingredient which is the 48 % acid equivalent of the isopropylamine salt of glyphosate) was used.

Acute toxicity tests

The acute toxicity tests were performed according to the US EPA procedure for the static non-renewal technique.[5] Fish were not fed 48 hours before starting and 96 hours during the experiment. Preliminary screening was carried out to determine the appropriate concentration range for testing the chemical. The tests consisted of a control and at least five concentration groups, three replicates per group, with ten fish in each replicate. At the beginning of the tests and every 24 hours, the symptoms and the number of dead fish were recorded. The results of the median lethality concentration (LC_{50}) at 24 h, 48 h, 72 h and 96 h were computed using the EPA probit analysis computer program. In the histopathological analysis, the tests consisted of control and treated (fish exposed to Roundup at the 96-h LC_{50}, 36ppm) groups, three replicates per group, with ten fish in each replicate. Every 24 hours from the beginning through to the end of the experiment (96 h), 2 fish from each aquarium were anesthetized with MS-222 (tricaine methan sulphonate). The organs (gills, liver and kidneys) were removed and prepared for histopathological observation. They were fixed in Bouin's fluid for 24 hours, washed with 70% ethanol and dehydrated through a graded series of ethanol. They were embedded in paraffin, sectioned at 4 - 5 μm thickness, stained with hematoxylin and eosin, and examined for abnormalities using an Olympus Vanox light microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data were expressed as means ± SD. A two-way analysis of variance was performed separately for each month, and also separately tested in each group. Least significant difference (LSD) was used for mean separation. The significance level was set at the probability level of $p < 0.05$.

Results

Acute toxicity tests

The results of the 24-h, 48-h, 72-h and 96-h LC_{50} values for Roundup in adult and young Nile tilapia were calculated by the probit method and presented in Table 1. The values of 24-h, 48-h, 72-h and 96-h LC_{50} for young tilapia were 17.5, 17.1, 16.9 and 16.8 ppm, respectively, and those for adult tilapia were 46.9, 44.4, 40.0 and 36.8 ppm, respectively.

Histopathological studies

Gills

Control group: No recognizable changes were observed in the gills of the control fish. Each gill consisted of a primary filament and secondary lamellae (Fig 1A). The primary lamellar epithelium was one or two cell layers thick. Chloride cells were visible along the primary lamellar epithelium,

Table 1. The LC_{50} values of Nile tilapia exposed to Roundup.

Animals	LC_{50} (ppm)			
	24 h	48 h	72 h	96 h
Adult Nile tilapia	46.9 ± 4.3	44.4 ± 3.8	40.0 ± 3.4 *	36.8 ± 3.2 *+
Young Nile tilapia	17.5 ± 15.3 a	17.1 ± 14.7 a	16.9 ± 14.2 a	16.8 ± 14.0 a

Note. * = The mean difference was significant in the row when compared the 24-h LC_{50} ($P < 0.05$)
+ = The mean difference was significant in the row when compared the 48-h LC_{50} ($P < 0.05$)
a = The mean difference was significant in the column ($P < 0.05$)

especially at the bases of secondary lamellae (Fig 1B). The secondary gill lamellae were composed of a single layer of epithelial cells supported by pillar cells (Fig 1B).

Treatment groups: Light microscopic study of the gills of Nile tilapia exposed to 36 ppm of Roundup (96-h LC_{50} value) for 24, 48, 72, and 96 hours showed several pathological changes and their frequencies increased with increasing time of exposure. After 24 h of Roundup exposure, filament cell proliferation was quantified by the height of the filament epithelium. The thickening of the primary lamellar epithelium appeared regular, similar to that of the control. After 48 h of exposure, the thickness of the primary lamellar epithelium was noticeably irregular (Fig 2A). Mild respiratory epithelial proliferation was observed when the epithelial layer was slightly hyperplastic. Edema with lifting of secondary lamellar epithelium and leukocyte infiltration was observed (Fig 2A). After 72 h of exposure, the thickness of the primary lamellar epithelium was still irregular. Groups of erythrocytes were observed at the secondary lamellae (Fig 2B). Similar changes remained after 96 h of exposure, with more pronounced thickness of the primary lamellar epithelium (Fig 2C). Clubbing of lamellae was concentrated at the tips of the secondary lamellae (Fig 2D).

Liver

Control group: The liver histology of control fish revealed the typical parenchymatous appearance. At the light microscopic level, the liver was divided into irregularly shaped lobules separated by the hepatopancreas and bile duct (Fig 3A). The liver was made up of hepatocytes that were polygonal cells with a central spherical nucleus and a densely stained nucleolus (Fig 3B).

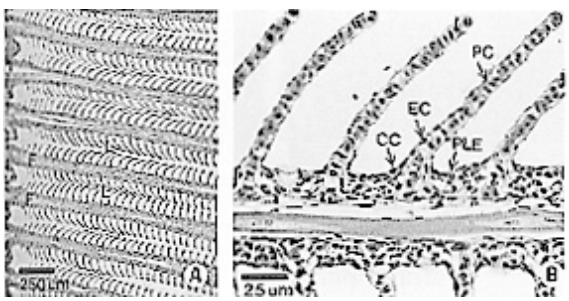

Fig 1. Transverse section of Nile tilapia gills in control group.
A. Low magnification showing normal appearance of primary filament (F) and secondary lamellae (L).
B. High magnification showing normal appearance of primary lamellar epithelium (PLE), chloride cell (CC), epithelial cell (EC), and pillar cell (PC).

Treatment groups: After 24 h of exposure, the hepatocytes began to swell and slight infiltration of leukocytes was observed (Fig 3C, D). After 48 h of exposure, the hepatocytes were still swelling. They showed extensive pyknosis, and involution exhibiting darkly stained specks of necrotic nuclei, as well as mild infiltration of leukocytes. There was some degeneration of the cell membrane and vacuolation in the cytoplasm (Fig 4A, B). After 72 h of exposure, there were large vacuoles in the cytoplasm, the nuclei continued to be pyknotic, and moderate infiltration of leukocytes was observed (Fig 4C, D). After 96 h of exposure, more notable detrimental changes were observed. There was a severe infiltration of leukocytes. The hepatocytes showed extensive pyknotic nuclei and large vacuoles in many areas (Fig 4E, F).

Kidney

Control group: No recognizable changes were observed in the kidney of the control fish (Fig 5A). At the light microscopic level, the renal corpuscle was composed of the glomerulus and Bowman's capsule (Fig 5B). The first proximal tubule (P1) was composed of cuboidal or low columnar cells with a well-developed brush border containing vacuoles and round basal nuclei (Fig 5C).

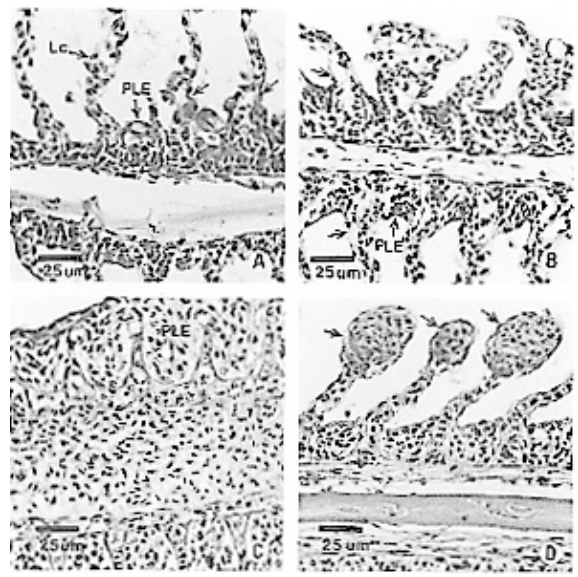

Fig 2. Transverse section of Nile tilapia gills exposed to Roundup at 48h, 72h and 96h.
A. The 48-h exposure showing irregular thickening of primary lamellar epithelium (PLE), edema (arrows) and a slight infiltration of leukocytes (Lc).
B. The 72-h exposure showing the thickening of primary lamellar epithelium (PLE), several areas of edema (arrows), lamellae packed with erythrocytes (*).
C, D. The 96-h exposure showing severe thickening of primary lamellar epithelium (PLE in C) and clubbing at the tips of the secondary lamellae (arrows in D).

Treatment groups: After 24 h of exposure, the fish had renal lesions of varying degrees. Some glomeruli were collapsed or distorted. The epithelial cells of the first proximal tubule contained small vacuoles. Some of the cells were necrotic with small pyknotic nuclei (Fig 6A). After 48 h of exposure, some of the P1 epithelial cells still had small vacuoles. Some exhibited small hyaline droplets (Fig 6B). After 72 h of exposure, they had extensive vacuoles and were swollen with pyknotic nuclei (Fig 6C). After 96 h of exposure, the epithelium of many of the tubules had become exfoliated. The cytoplasm was pale and the cells appeared swollen. Pyknotic nuclei were observed in these tubules (Fig 6D).

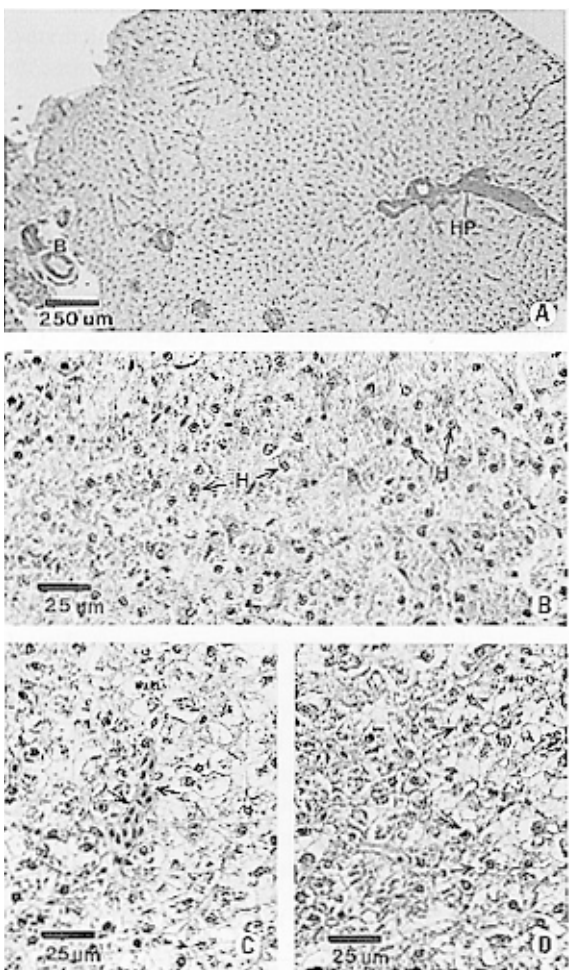

Fig 3. Light micrographs of a transverse section of Nile tilapia liver in control and 24-h exposure.
A. Low magnification showing irregular-shaped lobules of the liver with bile duct (B) and hepatopancreas (HP).
B. High magnification showing hepatocytes (H) with a central spherical nucleus (arrows).
C, D. The 24-h exposure showing a slight infiltration of leukocytes (arrows in C), and swelling of hepatocytes (arrows in D).

Discussion

Roundup is one of the most widely used herbicides, and its use is increasing rapidly with GM Roundup-tolerant crops. It is considered to be persistent and mobile in soil and water, and is known to be one of the most common terrestrial and aquatic contaminants.[6] The recommended field application rates usually range between 1500 and 2000 ppm per rai. Frank[7] reported that glyphosate had been detected in a watershed 4 months after application. After a year, 0.1 ppm of glyphosate was still found in the sediment of a farm pond.[8] The use of glyphosate as a herbicide may result in the presence of residues in surface waters and run-off from agricultural areas. It can be detected in most aquatic systems, including streams (up to 150 ppm), pond (up to 250 ppm), as well as surface water (up to 25 ppm).[1] Thus, glyphosate concentrations measured in the field may exceed the values recommended for

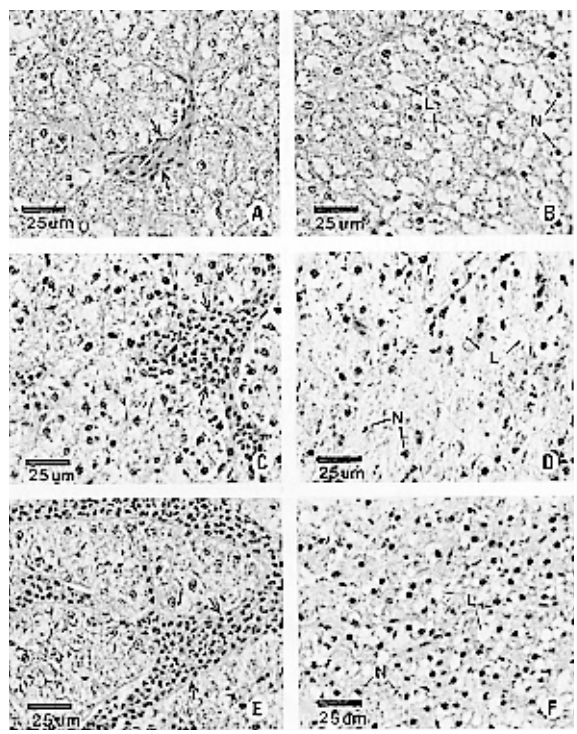

Fig 4. Light micrographs of Nile tilapia liver in fish exposed to Roundup at 48 h, 72 h and 96 h.
A, B. The 48-h exposure showing mild infiltration of leukocytes (arrows). The hepatocytes are still swelling with some pyknotic nuclei (N) and a few small lipid vacuoles (L).
C, D. The 72-h exposure showing moderate infiltration of leukocytes (arrows), pyknotic nuclei (N) and lipid vacuoles (L).
E, F. The 96-h exposure showing severe infiltration of leukocytes (arrows), pyknotic nuclei (N) and lipid vacuoles (L).

drinking water by the EC guideline 98/83/EC (the parametric values PVs = 0.1 mg/L)[9], by WHO (the guideline values GVs = 0.1 mg/L)[10], and by the US EPA (the maximum contamination levels MCLs = 0.7 mg/L).[11] So, one must be concer ned with monitoring or surveillance of Roundup residues and/or their impacts in the environment.

The LC_{50} values of Roundup vary widely from 2 ppm to 55 ppm have been reported depending on the species of fish and test conditions.[1] Mitchell et al[2] reported the results of aquatic toxicity testing of Roundup using three different fish species (rainbow trout, chinook salmon, and coho salmon) and different water types (dechlorinated, dechlorinated and reconstituted, and natural lake water), pH (6.1-7.7), hardness (4.5-85.0 mg/L as $CaCO_3$), and conductivity (12-132 mmhos/ cm). The results showed that the 96-h LC_{50} values varied from 15-26 mg/L, depending on the fish species, water characteristics, pH and hardness. At the same time, Servizi et al[3] reported that the 96-h LC_{50} values of Roundup were 28.0 and 42.0 mg/L for rainbow trout and coho salmon, respectively. Abdelghani et al[12] evaluated the acute toxicity of Roundup using three species of freshwater organisms (crawfish, channel catfish and bluegill sunfish). The 96-h LC_{50} values were 4.9, and 4.4 mg/L for catfish and bluegill sunfish, respectively.

In the present study, the results of 24-h, 48-h, 72-h, and 96-h LC_{50} values for adult tilapia were 46.9, 44.4, 40.0 and 36.8 ppm, respectively. The corresponding LC_{50} values for young tilapia were 17.5, 17.1, 16.9, and 16.8 ppm, respectively. The LC_{50} values obtained from the present study fell within the concentration ranges reported in the previous studies. The LC_{50} values at different time intervals in adult Nile tilapia exposed to Roundup were statistically different. But they were not statistically different in young Nile tilapia exposed to Roundup at different time intervals. In addition, these values for the two age groups were statistically different at each time interval, indicating that the young fish were more sensitive to Roundup than the

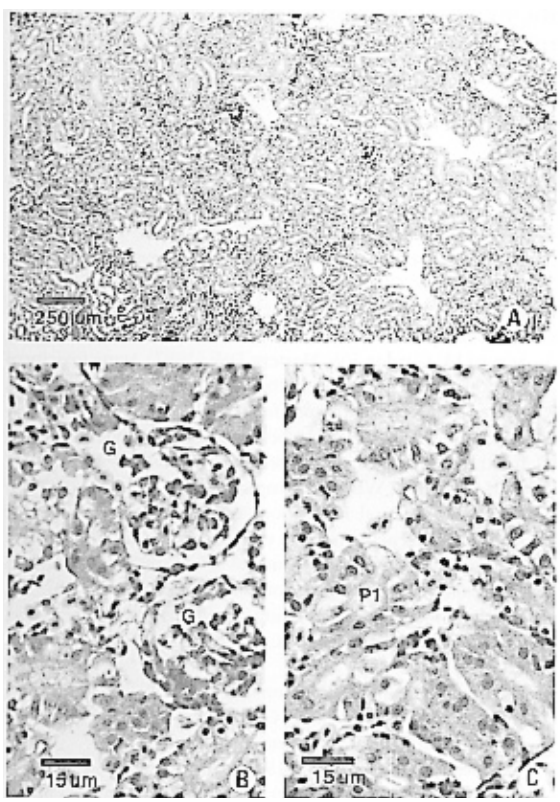

Fig 5. Light micrographs of a transverse section of Nile tilapia kidney in the control group.
A. Low magnification showing normal appearance of kidney.
B. High magnification showing glomerulus (G),
C. High magnification showing the first proximal tubule (P1).

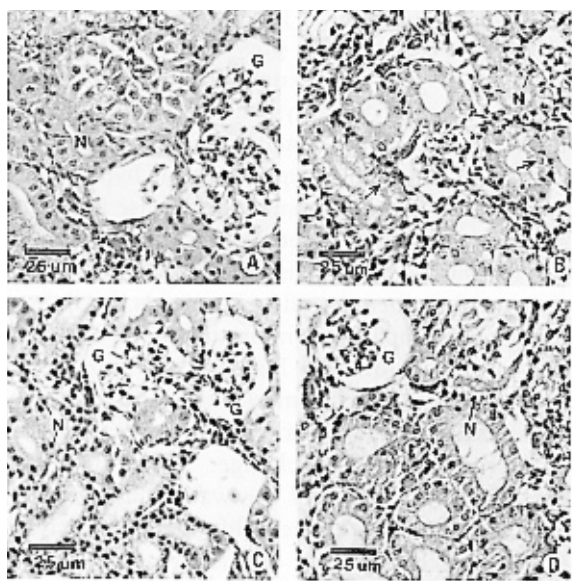

Fig 6. Light micrographs of Nile tilapia kidney in fish exposed to Roundup at 24 h, 48 h, 72 h and 96 h.
A. The 24-h exposure showing glomerulus distortion and broadening of Bowman's capsule (G). Notice small vacuoles (*) and some pyknotic nuclei (N) in the P1 epithelial cells.
B. The 48-h exposure showing hyaline droplets (arrows), small vacuoles (*) and pyknotic nuclei (N) in the P1 epithelial cells.
C. The 72-h exposure showing glomerulus distortion and broadening of Bowman's capsule (G). Notice the extensive vacuoles (*) in the swollen P1 epithelial cells with pyknotic nuclei (N).
D. The 96-h exposure showing glomerulus distortion and broadening of Bowman's capsule (G). The epithelium of the tubules has become exfoliated (*) and swollen with pyknotic nuclei (N).

adult fish. This was in agreement with previous studies reporting that fish species are the most sensitive to aquatic pollutants during the early life stages. Folmar et al[13] reported that the exposure of early life-stages of rainbow trout and channel catfish to Roundup showed that the egg stage was the least sensitive for both species. Toxicity of Roundup increased in the sac fry and early swim-up stages, but decreased in the fingerling stage as the fish grew larger.

The literature on histopathological effects of Roundup on fish is extremely sparse. Neskovic et al[14] conducted subacute toxicity tests (14 days) of sublethal glyphosate concentrations on histopathological changes of carp organs such as gills, liver and kidneys. In the gills of fish exposed to glyphosate, epithelial hyperplasia and subepithelial edema were found. Congestion of few sinusoids and, at some places, signs of early fibrosis in the liver were recorded. No histopathological changes were found in the kidney.

In fish exposed to 36 ppm Roundup concentration (96-h LC_{50}) in the present study, the major changes in gills were edema, epithelial lifting, thickening of the primary lamellar epithelium, and fusion of secondary lamellae. Histopathological changes of gills such as hyperplasia and hypertrophy, epithelial lifting, aneurysm and increase in mucus secretion have been reported after the exposure of fish to a variety of noxious agents in the water, such as pesticides, phenols and heavy metals.[15] All these lesions may impair respiratory function. Filament cell proliferation and lamellar pavement cell hypertrophy reduce the interlamellar space and may cause a complete lamellar fusion reducing the total surface area for gas exchange. Otherwise, they increase the distance of the water-blood barrier, which together with epithelial lifting and the increase in mucus secretion may drastically reduce the O_2 uptake and, if the damaging agent is not removed, can lead to the rupture of blood vessels with small hemorrhage focus.[15]

In the present study, the liver of Nile tilapia exposed to 36 ppm Roundup concentration showed an infiltration of leukocytes, increasing hepatocyte size with pyknotic nuclei, and presence of vacuoles. In the study of Risbourg and Bastid[16], the exposure of fish to atrazine herbicide had increased in the size of lipid droplets, vacuolization in the liver. The most frequent encountered types of degenerative changes are those of hydropic degeneration, cloudy swelling, vacuolization, and focal necrosis. Pyknosis, karyorhexis, and karyolysis have been reported in cases of severe intoxication. The vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation.[17]

In the present study, the kidney of Nile tilapia exposed to 36 ppm Roundup concentrations showed dilation of Bowman's space and accumulation of hyaline droplets in the tubular epithelial cells of the first proximal tubule. Oulmi et al[18] studied the effects of linuron herbicide on the rainbow trout (*Oncorhynchus mykiss*). Their results showed small cytoplasmic vacuoles, nuclear deformation in the epithelium of the first and second segments of the proximal tubule. The deposition of brightly stained hyaline droplets within the cells of the proximal tubules was also frequent. This often displaced the nucleus, and represented protein that had been reabsorbed from the glomerular filtrate. Because the excretion of divalent ions is a major function of the renal tubular epithelium, pollution with heavy metals or pesticides would be highly likely to affect these cells. The presence of hyaline droplets in renal tubules has been suggested to be an indicator of renal toxicity for a variety of chemicals, including pesticides.[19]

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