



Effects of glyphosate in common carp: Histopathological and immunohistochemical study

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Article information

Article history:

Received October 23, 2022

Accept March 29, 2023

Available online June 11, 2023

Keywords:

Organophosphorus

Cyprinus carpio

IL-6

IL-12

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Abstract

We aimed in current study to determine the LC₅₀ of glyphosate in common carp fish (*Cyprinus carpio* L.) within 24 hours in addition to study the pathological lesions of sublethal concentration of glyphosate (25 and 50% of LC₅₀) in kidney and liver tissues. A total of 100 fish used to determine the LC₅₀ of glyphosate using Trevan method (Curve protocol) within 24 hours, in addition to 75 fish used to study the histopathological lesions caused by 25 and 50% of LC₅₀ in common carp after 5, 15 and 25 days of exposure. The result of current study showed that the LC₅₀ of glyphosate in common carp was 26.151 mg/L within 24 hours, in accordance with this result the 25 and 50% of LC₅₀ was 6.54 and 13.08 mg/L, respectively. The histopathological lesions revealed that both kidney and liver showed degenerative and necrotic changes which associated with infiltration of inflammatory cells and interstitial hemorrhages, also the liver sections showed hyperplasia of bile canaliculi and collagen fiber deposition within portal area, generally the histopathological lesions were more intensive and advanced in group exposed to 50% of glyphosate LC₅₀ more than that recorded in group treated with 25% of glyphosate LC₅₀. In conclusion, common carp fish (*Cyprinus carpio* L.) showed serious histopathological lesions after exposure to sublethal concentration of glyphosate, these lesions are life threatening and can cause high mortality in higher concentration.

DOI: [10.33899/ijvs.2023.136627.2601](https://doi.org/10.33899/ijvs.2023.136627.2601), ©Authors, 2023, College of Veterinary Medicine, University of Mosul.

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Introduction

Glyphosate is a polar of organophosphorus compound dissolved in water intensively, composed at first time in 1970 by Martin Henry during his working in Swiss Pharmaceutical, and accepted as herbicides in 1974 (1). The annual production in 1975 reach 240 ton, this production where increased to be 240,000 ton in 2014, and considered as the most commonly used herbicides in USA (2). Glyphosate is a wide spectrum herbicide used to eliminate the annual herbs (3), also used in control the over vegetation in water channels and fish ponds (4). Many studies showed a significant toxic effect of glyphosate in humans after glyphosate swelling which cause nausea, vomiting and unconsciousness with digestive and respiratory disturbance

(5,6), in addition, glyphosate direct contact with eye in rabbits cause local irritation and temporary blindness (7). In 2021 a report conducts on mice showed that the continuous oral administration led to renal failure due to losing the epithelial cell covering renal tubules and high rate of apoptosis in these cells (8). In Zebra fish (*Diplodus cervinus*) exposed to glyphosate at 85 mg/L for 72 hours that added to pond water cause increase in mortality rate due to necrotic changes in renal tissues (9,10). In addition, adding glyphosate to pond water to a dose 2.5, 5 and 10 mg/L of water to grass carp lead to hyperplasia of gill lamella with edema within two days (11). Another study showed that exposure of Pava fish to 0.1, 1 and 5 mg/L in water cause depletion in glycogen in the hepatocytes in addition to increase in degeneration of the affected hepatocytes (12).

We aimed in current study to determine the median lethal concentration (LC₅₀) of glyphosate in common carp (*Cyprinus carpio* L.) in 24 hours, and study the histological and immunohistochemical (IHC) changes associated with sublethal concentration of glyphosate.

Materials and methods

Estimation the LC₅₀ of glyphosate

A total of 100 common carp fish (weight 100±5 grams) was used in current experiment, these fish was divided into ten groups (10 fish/group), each fish group kept in glass pond, the water temperature at 22±2°C and the dissolved concentration kept at 8±1 mg/L, and the pH at 7.4±2 in all experimental period. A calculated dose of glyphosate (1, 2, 5, 10, 15, 20, 25, 50, 60 and 120 mg/L) added to each pond after dilution in secondary containers, the mortality rate was recorded within 24 hours after first exposure. The collected data (dose concentration, total number of fish in each pond, and the mortality number of fish for each concentration) was used to estimate the LC₅₀ using Trevan methods (curve methods) within 24 hours (13).

Experimental design

A total of 75 common carp fish (weight 100±5 grams) was used in current study, these fish was divided into three groups (25 fish/group), each fish group kept in glass pond, the water temperature at 22±2°C and the dissolved concentration kept at 8±1 mg/L, and the pH at 7.4±2 in all experimental period. The first group consider as control group, kept without any treatment in all experimental time. The second group exposed to 25% of the glyphosate LC₅₀. The second group exposed to 50% of the glyphosate LC₅₀. At each 5, 15, and 25 days of experimental a five fish from each group was euthanized, a tissue samples from liver and posterior kidney were collected and fixed in 10% neutral buffered formalin (NBF) to be included in the histopathological and immunohistochemical analysis (14).

Histopathological protocol

Tissue samples fixed in 10% NBF was trimmed to have a represented sample, these tissue samples were dehydrated with ascending concentration of ethyl alcohol, cleared in xylene, infiltrated and blocked with host liquid paraffin wax at 57±1°C (15). The paraffin block sectioned at 5±1 µm and lifted on glass slide, then stained with Harris' hematoxylin and alcoholic eosin for routine staining (14).

IHC staining

A section at 4 µm thickness from paraffin block were obtained and loaded on charged slides, the section where the primary antibody was polyclonal anti-rabbit IL-6 at 1:400 dilution (Cat. No. E-AB-40021, Elabscience, USA) and polyclonal anti-rabbit IL-12B at 1:300 dilution (Cat. No. E-

AB-13323, Elabscience, USA), the manual staining protocol for IHC was applies (16,17).

Results

Estimation the glyphosate LC₅₀

The result of current study showed that the LC₅₀ for glyphosate in common carp fish was 26.151 mg/L of pond water within 24 hours (Figure 1). Depending on these results the doses that will be used in the experimental part of histopathological study will be for 25% of LC₅₀ is 6.54 mg/L of pond water and the 50% of LC₅₀ is 13.08 mg/L of pond water.

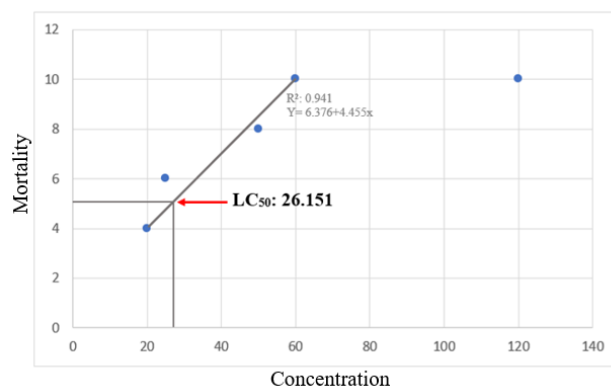


Figure 1: Curve for determination the LC₅₀ of glyphosate in common carp.

Kidney histopathological changes

The result of current study showed that after five days of experiment the glyphosate toxicity cause infiltration of inflammatory cells in the hemopoietic tissues with vacuolar degeneration in the epithelia cells lining renal tubules in group treated with 25% of LC₅₀ of glyphosate, a similar lesion was recorded in group treated with 50% of LC₅₀ of glyphosate in addition presence of cogaulative necrosis in other tubules in compare with control group. After 15 days cogaulative necrosis was shown in the glomerular tuft and in renal tubules in addition to cloudy cell swelling in group treated with 25% of LC₅₀ of glyphosate, while in group treated with 50% of LC₅₀ of glyphosate infiltration of mononuclear inflammatory cells in the hemopoietic tissues with interstitial hemorrhages was observed in compare to control group. While after 25 days of experiment, the common carp fish lesion in renal tissue shown vacuolar degeneration in glomerular tuft and in renal tubules, also infiltration of mononuclear inflammatory cell in the hemopoietic tissue in group treated with 25% of LC₅₀ of glyphosate, a similar lesion was observed in group treated with 50% of LC₅₀ of glyphosate in addition to deposition of amorphous eosinophilic materials within the distended renal tubules (Figure 2).

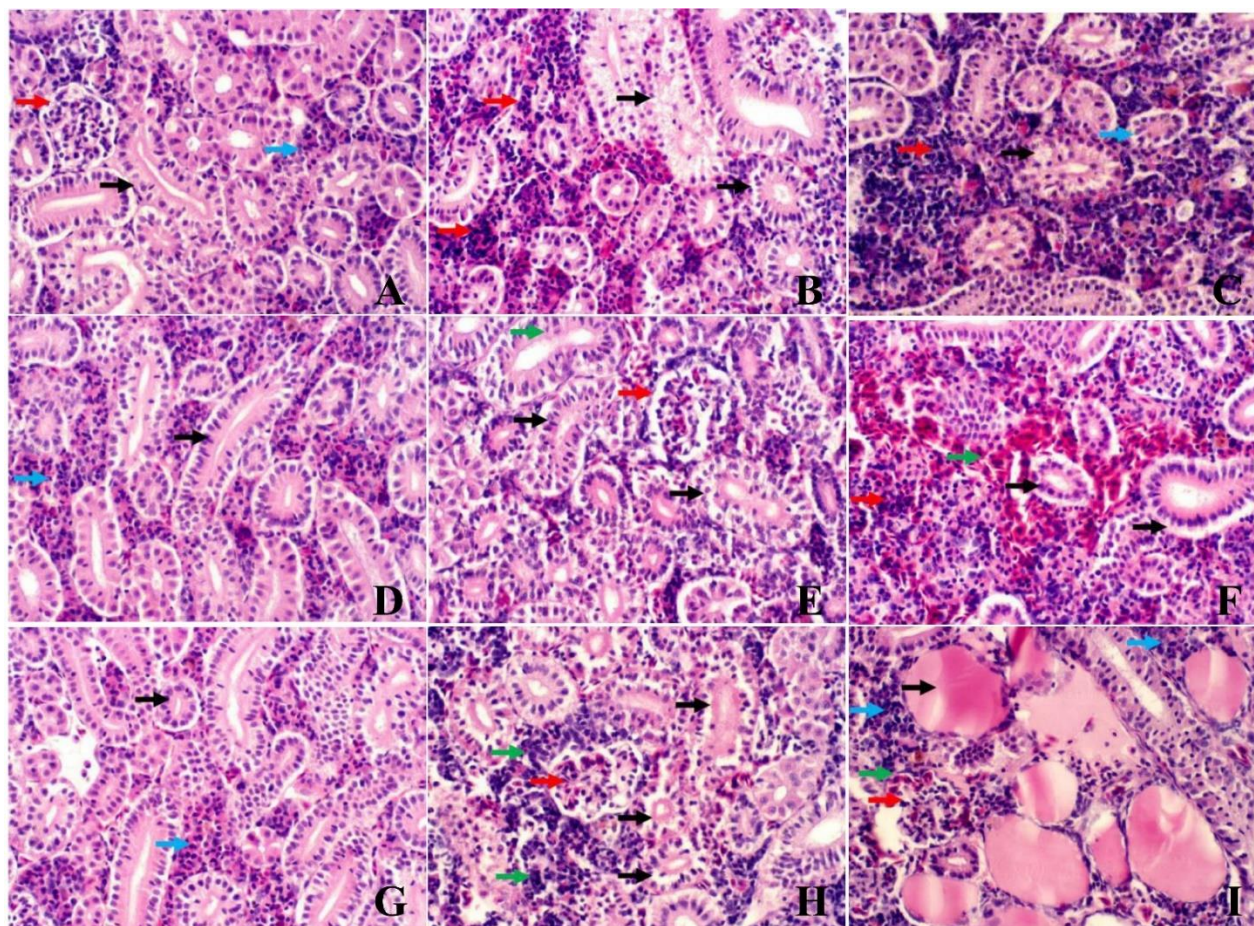


Figure 2: Posterior kidney (*Cyprinus carpio*), [A] control group, 5 days. Normal kidney histology represented by glomeruli (red arrow), renal tubules (black arrow), hemopoietic tissue (blue arrow). [B] 25% glyphosate LC₅₀ group, 5 days. Infiltration of mononuclear inflammatory cells in the hemopoietic tissues (red arrow), vacuolar degeneration in epithelia cell lining renal tubules (black arrow). [C] 50% glyphosate LC₅₀ group, 5 days. Infiltration of mononuclear inflammatory cells in the hemopoietic tissues (red arrow), vacuolar degeneration in epithelia cell lining renal tubules (black arrow), and cogaulative necrosis (blue arrow). [D] control group, 15 days. Normal kidney histology represented by renal tubules (black arrow), hemopoietic tissue (blue arrow). [E] 25% glyphosate LC₅₀ group, 15 days. Cogaulative necrosis in glomerular tuft (red arrow), and in renal tubules (black arrow), with cloudy cell swelling lead to stenosis of the tubule (green arrow). [F] 50% glyphosate LC₅₀ group, 15 days. Infiltration of mononuclear inflammatory cells in the hemopoietic tissues (red arrow), cogaulative necrosis in epithelia cell lining renal tubules (black arrow), with interstitial hemorrhages (green arrow). [G] control group, 25 days. Normal kidney histology represented by renal tubules (black arrow), hemopoietic tissue (blue arrow). [H] 25% glyphosate LC₅₀ group, 25 days. Vacuolar degeneration in glomerular tuft (red arrow), and in renal tubules (black arrow), with infiltration of mononuclear inflammatory cell in the hemopoietic tissue (green arrow). [I] 50% glyphosate LC₅₀ group, 25 days. Destruction and sloughing of the glomerular tuft (red arrow), deposition of amorphous eosinophilic material in large vacuoles (black arrow), interstitial hemorrhages (green arrow), and infiltration of mononuclear inflammatory cells (blue arrow). H&E, 400x.

Liver histopathological changes

The result of liver sections showed that in 25% glyphosate LC₅₀ group at 5 days have vacuolar degeneration in different size and shape within the cytoplasm of affected hepatocytes, while in 50% glyphosate LC₅₀ group showed presence of cogaulative necrosis in hepatocytes with vacuolar disturbance represented by central vein congestion and interstitial hemorrhages in compare with control group.

After 15 days the 25% glyphosate LC₅₀ group showed increase in the tensity of vacuolar degeneration with increase in size of formed vacuoles in the affected hepatocytes with signs of cellular disturbance represented by hyperplasia of the bile cuniculi with deposition of collagen fibers around portal area. In 50% glyphosate LC₅₀ group, advance stages of cogaulative necrosis were recorded which is widely distributed in hepatocytes these necrotic changes associated

with increase in numbers of Kupfer cells with hyperplasia in the portal area specially hyperplasia of bile cuniculi in compare with control group. While after 25 days the 25% glyphosate LC₅₀ group showed massive distribution of vacuolar degeneration in the hepatocytes, and these cells have either one large or multiply vacuoles, in addition to presence of vacuolar changes in the epithelial cells that lining the bile cuniculi which suffer already from hyperplasia, and presence of interstitial hemorrhages. In 50% glyphosate LC₅₀

group a more advance lesions of inflammation were recorded specially at the sifting from acute to chronic lesions, which represented by massive fibrosis in porta areas due to deposition of collagen fiber with vacuolation in the epithelial cells that lining bile cuniculi, with wide distribution of interstitial hemorrhages associated with infiltration of inflammatory cells between hepatocytes and around portal area, with hyperplasia of bile cuniculi comparing with control group (Figure 3).

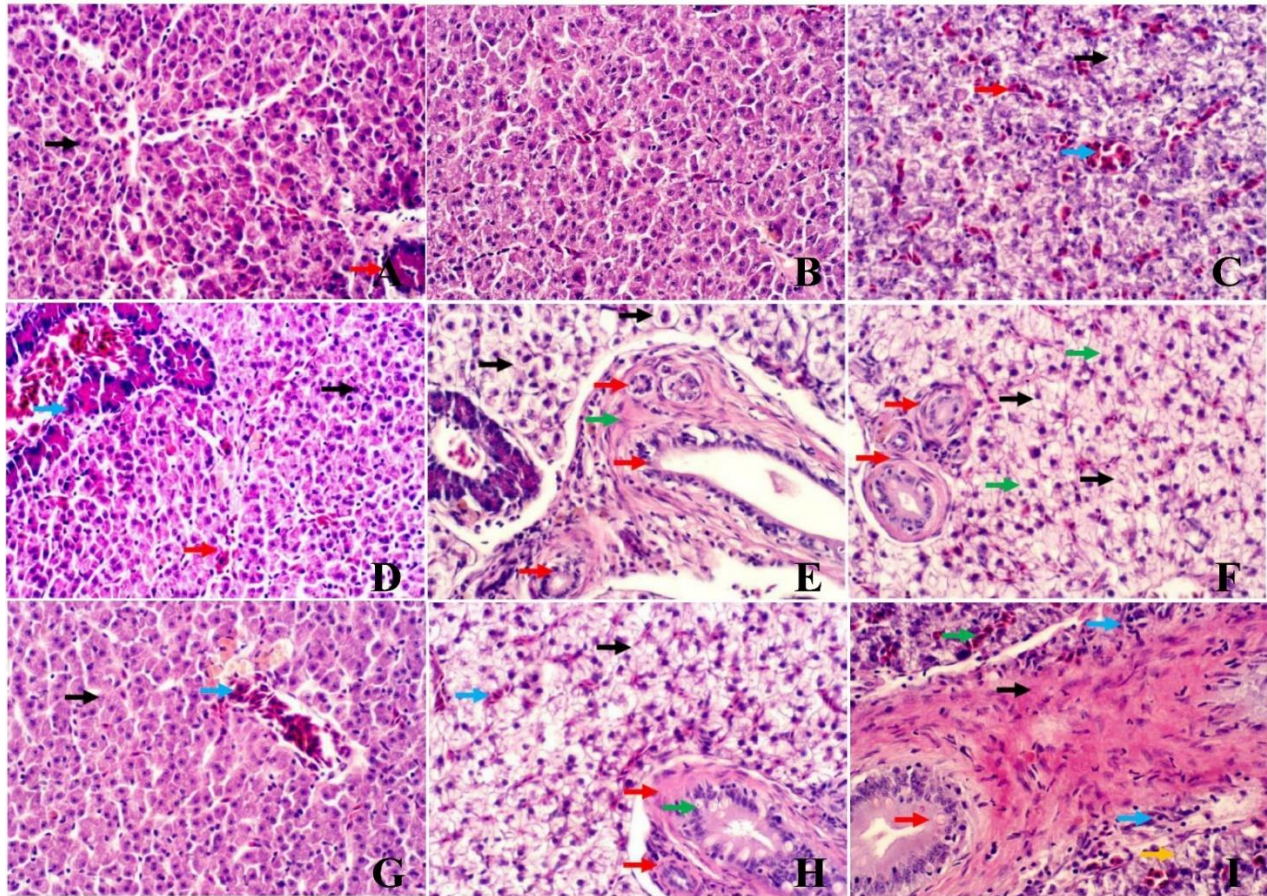


Figure 3: Posterior lobe of liver (*Cyprinus carpio*), [A] control group, 5 days. Normal hepatic histology represented by hepatocytes (black arrow), pancreatic tissue (blue arrow). [B] 25% glyphosate LC₅₀ group, 5 days. Vacuolar degeneration in the affected hepatocytes which appears as small size in different shape of clear vacuoles. [C] 50% glyphosate LC₅₀ group, 5 days. Cogaulative necrosis in the affected hepatocytes (black arrow), congestion of the central vein (blue arrow), with hemorrhages (red arrow). [D] control group, 15 days. Normal hepatic histology represented by hepatocytes (black arrow), pancreatic tissue (blue arrow), and hepatic sennosides (red arrow). [E] 25% glyphosate LC₅₀ group, 15 days. Vacuolar degeneration in the hepatocytes (black arrow), deposition of collagen fibers around bile cuniculi (blue arrow), hyperplasia of bile cuniculi (red arrow). [F] 50% glyphosate LC₅₀ group, 15 days. Cogaulative necrosis in the affected hepatocytes (black arrow), increase in number of Kupfer cells (green arrow), hyperplasia of bile cuniculi (red arrow). [G] control group, 25 days. Normal hepatic histology represented by hepatocytes (black arrow), pancreatic tissue (blue arrow). [H] 25% glyphosate LC₅₀ group, 25 days. Vacuolar degeneration in the hepatocytes (black arrow), with interstitial hemorrhage (blue arrow), hyperplasia of bile cuniculi (red arrow), with vacuolar degeneration in the epithelia cells lining bile cuniculi (green arrow). [I] 50% glyphosate LC₅₀ group, 25 days. Fibrosis in portal area represented by collagen fibers (black arrow), infiltration of inflammatory cells (blue arrow), vacuolar degeneration in the epithelia cells lining bile cuniculi (red arrow), interstitial hemorrhages (green arrow), and cogaulative necrosis in hepatocytes (arrow). H&E, 400x.

IL-6 and IL-12 IHC immunohistochemistry

The result of current study showed that the expression of IL-6 was in its high expression in the 5 days after exposures and reduced dramatically till the 25 days which in its lowest

expression (Figure 4), on other hand the expression of IL-12 was in its lowest expression in the 5 days after exposure and increase rapidly till its reach highest expression in 25 days after exposure in both kidney and liver tissue (Figure 5).

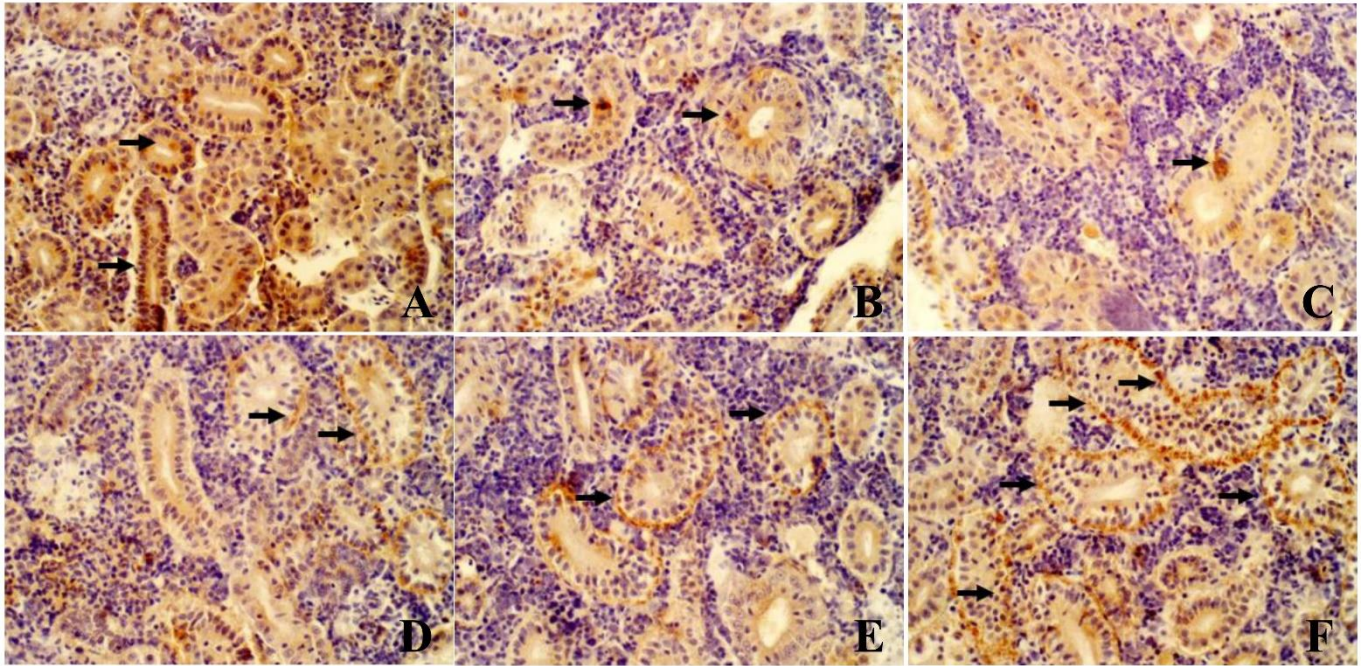


Figure 4: Posterior Kidney (*Cyprinus carpio*), [A] 25% glyphosate LC₅₀ group, 5 days. Strong positive reaction with IL-6 antibody appears as golden brown cytoplasmic granulose in the cytoplasm of epithelial cells lining renal tubules (black arrow). [B] 50% glyphosate LC₅₀ group, 15 days. Positive reaction with IL-6 antibody appears as golden brown cytoplasmic granulose in the cytoplasm of epithelia cells lining renal tubules in the cytoplasm of epithelial cells lining renal tubules (black arrow). [C] 50% glyphosate LC₅₀ group, 25 days. Weak positive reaction with IL-6 antibody appears as golden brown cytoplasmic granulose in few epithelial cells (black arrow). [D] 50% glyphosate LC₅₀ group, 5 days. Positive reaction with IL-12 antibody appears as golden brown cytoplasmic granulose in the basement membrane of epithelial cells (black arrow). [E] 25% glyphosate LC₅₀ group, 15 days. Strong positive reaction with IL-12 antibody appears as golden brown cytoplasmic granulose in the basement membrane of epithelial cells (black arrow). [F] 50% glyphosate LC₅₀ group, 25 days. Strong positive reaction with IL-12 antibody appears as golden brown cytoplasmic granulose in the basement membrane of epithelial cells (black arrow). IHC, 400x.

Discussion

The current study recorded the LC₅₀ of glyphosate was 26.151 mg/L of pond water within 24 hours in common carp (*Cyprinus carpio*), these result was showed higher than recorded by study conducted by Al-Rudainy and Azeez (18) in Iraq, were they found that the LC₅₀ was 4.15 mg/L of pond water within 24 hours in *Barbus sharpeyi* fish, this high concentration of glyphosate to induce 50% mortality in common carp fish can be due to the high resistance ability of this type of fish to these toxic chemicals, in addition, common carp prefer globally due to high adaptation ability in different water environments (19).

Other study conducted by Vajargh *et al.* (20) showed that the LC₅₀ to glyphosate in common carp was 166.161 mg/L of pond water within 24 hours, this result is too higher than

that recorded in current study, this can be due to the temperature of pond's water, which play important role in the increasing the toxic effect of poisonous materials, which is higher in current study to that used in Vajargh *et al.* study (20).

The histopathological examination of liver and kidney tissue revealed that the using of 25% of glyphosate LC₅₀ cause degenerative changes with scant infiltration of inflammatory cells in addition to edema, and all these changes can be reversable and the tissue return to its normal function and histology after removal of causative agent, in contrast the 50% of glyphosate LC₅₀ cause necrotic changes mostly cogaulative type, with focal and diffuse infiltration of inflammatory cells in addition to hyperplasia of bile cuniculi and fibrosis in portal area.

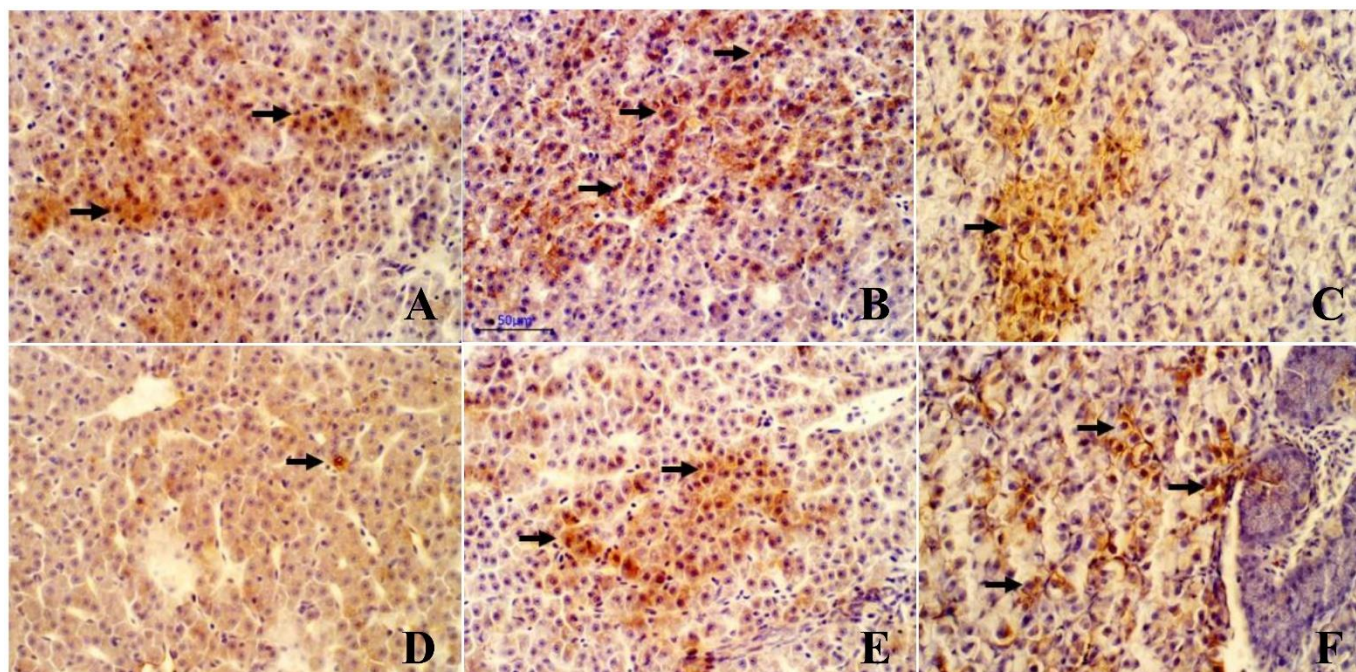


Figure 5: Posterior lobe of liver (*Cyprinus carpio*), [A] 25% glyphosate LC₅₀ group, 5 days. Strong positive reaction with IL-6 antibody appears as golden brown cytoplasmic granule in the hepatocytes (black arrow). [B] 25% glyphosate LC₅₀ group, 15 days. Strong positive reaction with IL-6 antibody appears as golden brown cytoplasmic granule in the affected hepatocytes (black arrow). [C] 50% glyphosate LC₅₀ group, 25 days. Positive reaction with IL-6 antibody appears as golden brown cytoplasmic granule in the necrotic hepatocytes (black arrow). [D] 25% glyphosate LC₅₀ group, 5 days. Weak positive reaction with IL-12 antibody appears as golden brown cytoplasmic granule of the hepatocytes (black arrow). [E] 50% glyphosate LC₅₀ group, 25 days. Positive reaction with IL-12 antibody appears as golden brown cytoplasmic granule of hepatocytes (black arrow). [F] 50% glyphosate LC₅₀ group, 25 days. Positive reaction with IL-12 antibody appears as golden brown cytoplasmic granule of necrotic hepatocytes (black arrow). IHC, 400x.

The result of histopathological changes was agreed with other studies, in one study they exposed common carp (*Cyprinus carpio*) to 1.63 and 0.815 PPM of glyphosate for 26 days showed degenerative changes composed from vacuolar degeneration and coagulative necrosis in hepatocytes with pyknotic nucleus associated with infiltration of leukocytes between affected areas, also a varied degree of fatty degeneration which can be observed in different time of treatment (21).

Other study conducted on Nile Tilapia (*Oreochromis niloticus*) which exposed to 5 and 15 PPM of glyphosate in water for one months, their results showed swelling of hepatocytes with tiny to large vacuoles inside cytoplasm of affected hepatocytes, with time their infiltration of macrophages, in severe cases the vacuoles fusion with each other in one large vacuoles make hepatocytes similar to adipocytes, while in kidney the glomeruli showed few vacuolation other showed hypercellularity associated with coagulative necrosis or vacuolar degeneration in epithelial cells lining proximal renal tubules with increase in number of macrophages in the hemopoietic tissue (22).

All histopathological changes associated with glyphosate toxicity in both liver and kidney can be explained as a result of stoppage of oxidative cellular respiration that result in depletion or shutdown in ATP production that lead to loss of permeability control to cell membrane due to damage in the function of potassium - sodium pump lead to influx of fluid in side cell which appear as vacuolation of cell swelling, in addition to that loss of ATP supply within the cell leads to lose the ability of trapping proteolytic enzymes in lysosomes which release inside the cell and caused proteolysis to the cytoplasmic organelles lead to formation of typical coagulative necrosis lesion all these lesions normally showed accompanied with leucocytic infiltration in order to decrease the cellular damages and removing the necrotic tissues (23).

The IHC examination showed that the IL-6 was in high expression in the first five days the its expression decline with time, on other hand the expression of IL-12 was found in highest expression in the last days of experiments, this variation in expression comes from the functions of these two cytokines, as ever known the IL-6 act as acute proinflammatory cytokines its function to brings fluid and cell after the initial sublethal injury to cells then with time its

action will decreased and dimensioned as its serve in starring the acute inflammation, on other hand IL-12 known as chronic proinflammatory cytokines and also as anti-acute proinflammatory cytokines, that is mean it is act to stop the different stages of acute inflammation and initiate the chronic inflammatory response which have other characteristic cells and inflammatory pathway (23).

Conclusion

In conclusion, common carp fish (*Cyprinus carpio* L.) showed serious histopathological lesions after exposure to sublethal concentration of glyphosate, these lesions are life threatening and can cause high mortality especially in higher concentration.

Acknowledgements

The authors wish to express they're thanks to College of Veterinary Medicine, University of Mosul to support this study.

Conflicts of interest

No conflicts.

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تأثيرات الكليفوسات في اسماك الكارب: دراسة نسيجية وكيميائية مناعية نسيجية

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الخلاصة

هدفت الدراسة الحالية الى تحديد التركيز المميت الوسطي للكليفوسات في اسماك الكارب الاعتيادي وخلال ٢٤ ساعة مع دراسة التأثيرات المرضية للتعرض الى الجرعة دون المميتة الوسطية (٢٥ و ٥٠% من الجرعة المميتة الوسطية) في نسيج الكلية والكبد. تم استخدام ١٠٠ سمكة كارب اعتيادي لتقدير التركيز المميت الوسطي باستخدام طريقة تريغان (طريقة المنحنى)، كما استخدمت ٧٥ سمكة لغرض إجراء

عموماً كانت التأثيرات المرضية النسجية اشد ضرراً وأكثر تقدماً في المجموعة التي تعرضت الى ٥٠% من الجرعة المميّنة الوسطية عن التي سجلت في المجموعة التي تعرضت الى ٢٥% من الجرعة المميّنة الوسطية. في الاستنتاج، إن تعرض اسماك الكارب الاعتيادي للجرع دون المميّنة الوسطية من الكليفوسات سبب تغيرات مرضية نسجية خطيرة، وهذه التغيرات يمكن أن تكون مهددة للحياة وبالإمكان أن تسبب ارتفاع في نسبة الهلاكات في الجرع ذات التركيز الأعلى.

تجارب الدراسة الخاصة بالتأثيرات النسجية المرضية للجرع دون المميّنة الوسطية على نسيج الكبد والكلية. أظهرت النتائج أن الجرعة المميّنة الوسطية كانت ٢٦,١٥١ ملغرام/ لتر من ماء الحوض وخلال ٢٤ ساعة وان ٢٥ و ٥٠% من الجرعة المميّنة الوسطية كانت ٦,٥٤ و ١٣,٠٨ ملغرام/ لتر من ماء الحوض. أظهرت نتائج الفحص النسجي المرضي أن كل من الكبد والكلية أظهرتا تغيرات تنكسية وتخريره مع ارتشاح لخلايا التهابية والنزف الخلالي، وأظهرت مقاطع الكبد فرط تنسج الفقية الصفراوية مع ترسب الياف الكولاجين في الباحة البابية،