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Histological and Enzymatic Changes of the Liver in Common Carp (*Cyprinus carpio L.* 1758) After Chronic Exposure to A Mixture of Goldate and Alexander Pesticides

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Abstract

Pesticides are man-made chemical compounds, widely used in agriculture to control different types of pests, product crops and prevent diseases which damage them. This experiment used herbicide Goldate, insecticide Alexander and their mixture with different concentrations exposed to common carp in 40L glass aquaria. The results showed that there was a significant increase in the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) means value of mixture pesticides compared with control. The liver tissues of the fish exposure to pesticides groups G1, A1, M1 and M2 showed different degrees of jaundice, cellular swelling, necrosis hepatocytes and vascular degeneration of pancreatic acini and aggregation of bile pigments. These effects increased with the increase in pesticides concentrations and exposure time. These effects also increased when exposure was to more than one pesticide at the same time. The study aimed to evaluate the effects of two pesticides as mixture, study the chronic toxicity of Goldate, Alexander and their mixture by assessing ALT, AST and liver histological changes. This study concluded that the Goldate + Alexander mixture had adverse effects more than each one alone.

Keywords: *Cyprinus carpio*, Pesticides, Mixture, Histological, ALT, AST.

التغيرات النسيجية والإنزيمية للكبد في الكارب الشائع (*prinus carpio L.* 1758) بعد التعرض المزمن لمزيج من مبيدات Goldate و Alxander

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الخلاصة

مبيدات الآفات هي مركبات كيميائية من صنع الإنسان ، وتستخدم على نطاق واسع في الزراعة للسيطرة على أنواع مختلفة من الآفات ، وإنتاج المحاصيل ومنع الأمراض التي تضر بها. في هذه التجربة تم استخدام مبيدات الأعشاب (Goldate) والمبيدات الحشرية (Alexsander) ومزيجها بتركيزات مختلفة في أحواض زجاجية سعة 40 لتر تحتوي على أسماك الكارب الشائع. وجدت هذه التجربة زيادة معنوية في متوسط قيمة Alanine aminotransferase و Aspartate aminotransferase لخليط المبيدات مقارنة مع مجموعة السيطرة. أظهرت أنسجة الكبد عند تعرض الأسماك لمجموعات المبيدات الحشرية G1 و A1 و M1 و M2

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درجات مختلفة من اليرقان،التورم الخلوي، نخر خلايا الكبد، تنكس الأوعية الدموية في البنكرياس وتكدس الأصباغ الصفراوية. زادت هذه التأثيرات مع زيادة تراكيز مبيدات الآفات ووقت التعرض. كما زادت هذه التأثيرات عند التعرض لأكثر من مبيد في نفس الوقت. كما هدفت هذه الدراسة الى تقييم تأثير مبيدين على شكل خليط وذلك بدراسة السمية المزمنة لمبيدات Goldate و Alxander و خليطهما بتقييم انزيمات ALT و AST و التغير النسيجي للكبد، وقد اشارت نتائج الدراسة إلى أن المزيج (Goldate + Alxander) له تأثيرات أكثر من تأثير كل مبيد على حده.

Introduction

Pesticides are a big group of chemicals used in farming to prevent, repel and eliminate pests that may damage or disturb the health and growth of living organisms [1]. Pesticides are divided into four categories such as herbicides, insecticides, fungicides and rodenticides depending on their origin, structure or the mode/site of action [2]. Over time, pesticide residues accumulation in the environment components has led to the emergence of numerous ecological and health problems [3]. According to [4] the estimated 50% of the total surface water pollution received from agricultural sources, also pesticides had accumulation property and bioaccumulation in aquatic organisms tissues [5].

Fish have an important role in sustaining freshwater and marine ecosystems balance because it represents an essential component of it [6]. It is one of the common and widely distributed creatures in an aquatic environment that is susceptible to environmental pollution that can reflect the degree of the biological effects of environmental contaminants in waters. Blood parameters monitoring (both cellular and non-cellular) may be very useful in identifying early warning signals of pesticide poisoning [7]. Due to their diversity of habitat conditions, their movement and relatively long lifespan, fish are appropriate bio-indicators of chronic exposure [8][9].

In both laboratory and field studies, histological alterations are commonly employed as bio-indicators to evaluate the fish health which are exposed to pollutants. Therefore, histological changes can be employed as bio-indicator for evaluating the effects of many xenobiotic in organisms and reflect the overall health of whole ecosystem. Alterations in biochemical parameters could be utilized as a biomarker to assess the health state of aquatic creatures [10].

In order to measure the physiological health of fish, the examination of blood is a crucial approach. Measurements of haematological parameters can be used to identify alterations in a characteristic which exceeds its normal homeostatic limits [6]. Haematological parameters such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are used to indicate the effects of water pollution on fish [11].

The liver is the source of many metabolic pathways and activities. Therefore, exposure to pesticides can cause histological damage of hepatic tissues causing physiological disruption subsequently. The liver's oxidation, reduction, conjugation and hydrolysis activities are involved in the detoxification of xenobiotics. Previous research has indicated that xenobiotics could change the antioxidant defence system of an organism [12] [13][14][15]. This study aimed to evaluate the effects of two pesticides as mixture, study the chronic toxicity of Goldate, Alexander and their mixture by assessing ALT, AST and liver histological changes.

Materials and Methods

This experiment was carried out at the College of Science, University of Baghdad, Environmental and Pollution Laboratory on juvenile fish of common carp (*Cyprinus carpio*), weighing 60-80 gm. obtained from hatchery incubators in Al-Mahaweel city, Babylon, Iraq.

The first stage of laboratory included adaptation of the fish for 10-14 days in the aquaria for acclimatization [16]. The fish were distributed in glass aquaria filled with de-chlorinated water. During the whole experiment period fish were fed with 30% digestible protein 3 kcal/g digestible energy. The aquariums (40 Liter) were filled with de-chlorinated tap water (Table 1) prior to stocking fish throughout the experiment. Air pump connected to a capillary system continuously delivered air to each test aquarium. Only healthy fish were selected for this experiment [17]

Table 1: Results of physiochemical properties of aquaria

Physical and Chemical Properties	Range
Temperature (°C)	20- 28
Dissolved Oxygen (D.O) (mg/L)	5.5-7.9
Hydrogen ion Concentration (pH)	6.9-7.6
Electrical Conductivity (µs/cm)	850- 1220

Three concentration were prepared for every pesticide: Goldate (G), Alexander (A) and mixture (M) by following equation (C1V1= C2V2) [18], which were then exposed to fish for 3 and 6 weeks (Table 2).

Table 2: The concentrations of Goldate, Alexsander and mixture.

Groups Concentration	G mg/l	A mg/l	M mg/l
1 (1/10)	1.595	0.463	1.840+0.460
2 (1/100)	0.159	0.046	0.184+0.046
3 (1/200)	0.079	0.023	0.092+0.023
Control	0.0	0.0	0.0

Five fish were selected from each control and exposure groups during 3 - 6 weeks exposure period for hematological changes and liver tissue. Blood sampling was done by using syringes washed with heparin from the caudal vein and then blood samples were shifted to clotted tubes. For AST and ALT analysis the serum was obtained by centrifuged tubes at 3000 rpm for 10 min. Liver tissue samples were later taken from both pesticide exposure groups and control. The collected liver tissue was rinsed by saline solution (0.9% NaCl) to remove blood, mucus and debris stuck to tissues before using 10% formaldehyde used for fixation. After this, liver tissue was hydrated by using a dehydrating agent which graded alcohol series. The dehydrated tissues were cleared in xylene and then embedded in paraffin wax. A rotary microtome was used to cut tissue sections at 5 µm before staining by hematoxylin and eosin. Olympus optical microscope was used to observe the prepared slides and take pictures at 40× and 100× magnification [19].

The Statistical Analysis System SAS. 2018 [20] program was used to detect the effects of different factors in study parameters. Least significant difference (LSD) test (Analysis of Variation-ANOVA) was used to compare differences between means in this study.

Result and Discussion

Alanine Aminotransferase (ALT)

After 3 weeks into this experiment, a significant ($p \leq 0.05$) increase in the ALT mean value was found compared with control (C= 25.94 IU/L) in groups A1, A2, A3, M1 and M2 with values of 57.25, 72.05, 73.85, 55.10 and 45.85 IU/L respectively. On the other hand a

significant ($p \leq 0.05$) decrease was detected in the ALT mean in groups G1, G2, G3 and M3 which had values of 25.14, 29.10, 27.60 and 28.75 IU/L respectively (Table 3).

After 6 weeks, this experiment found a significant ($P \leq 0.05$) increase in the ALT mean value compared with control (C= 25.94 IU/L) in groups G3, A1, A2, A3, M1, M2 and M3 with the values of54.03, 73.03, 87.47, 88.81, 70.51, 71.73 and 70.71 IU/L respectively (Table 3).

The results showed a significant ($p \leq 0.05$) increase in the ALT mean value in groups G1, A1, M1 and M2 that had concentration of 1/10 of herbicide (Goldate), 1/10 of insecticide (Alexsander) and 1/10 and 1/100 of the mixture when comparing the differences of exposure times between 3 and 6 weeks.

This experiment found an increase in ALT value compared with the control group. The increase was directly proportionate with pesticides concentration and the exposure periods. Pesticides might alter activities of plasma hepatic and antioxidant enzymes, plasma protein levels and thyroid hormones depending on the water quality, toxicant type, fish species, and length of exposure [21][22].

Table 3: Effect of groups and period in ALT

Mean ± SE of ALT (IU/L)			
Group	**3 Weeks**	**6 Weeks**	**LSD Value**
C	25.94 ±0.65 B a	25.94 ±0.65 E a	2.55 NS
G1	25.14 ±0.26 B b	43.64 ±3.20 E a	13.83 *
G2	29.10 ±3.02 B a	43.58 ±2.74 DE a	17.54 NS
G3	27.60 ±0.30 B b	54.03 ±3.50 CD a	26.45 NS
A1	57.25 ±2.65 A b	73.03 ±2.05 AB a	14.42 *
A2	72.05 ±6.75 A a	87.47 ±1.61 AB a	29.86 NS
A3	73.85 ±10.25 A a	88.81 ±1.72 A a	44.72 NS
M1	55.10 ±10.20 A b	70.51 ±13.21 BC a	15.41 *
M2	45.85 ±10.75 A b	71.73 ±7.10 ABC a	25.41 *
M3	28.75 ±0.25 B a	70.71 ±10.18 BC a	43.81 NS
LSD value	18.17 *	17.74 *	---

ALT Mean value with different big-letters in the same column and small-letters in the same row are significantly different. * ($P \leq 0.05$).

Aspartate Aminotransferase (AST)

Three weeks into the experiment, significant ($p \leq 0.05$) increase in the AST mean value was detected compared with control (C= 28.16 IU/L) in groups G2, A1, A2, A3 and M2 with values of 16.45, 55.90, 60.90, 69.10 and 49.70) IU/L respectively. While there was no significant ($p \leq 0.05$) in the AST mean in groups G1, G3, M1 and M3 that had values of 31.35, 36.30, 45.05 and 44.06 IU/L respectively (Table 4).

After 6 weeks, a significant ($P \leq 0.05$) increase in the AST mean value was detected compared with control (C= 28.16 IU/L) in groups G2, A1, A2, A3, M1, M2 and M3 that had values of 63.45, 75.89, 73.12, 72.43, 56.55, 60.21 and 69.52 IU/L respectively. While there

was no significant ($P \leq 0.05$) in the AST mean in groups G1 and G3 which had values of 38.80 and 39.70) IU/L respectively (Table 4).

The results showed a significant ($p \leq 0.05$) increase in the AST mean values in G1, A1, M1 and M2 groups that had concentration of 1/10 of herbicide (Goldate), 1/10 of insecticide (Alexsander) and 1/10 and 1/100 of the mixture when comparing the differences of exposure times between 3 and 6 weeks.

Fish liver is one of the primary organs attacked by different types of pollutants and because of it has a vital role in the removal and detoxification of these substances, it shows alterations in biochemistry [23]. Increase in AST mean value compared to the control group with increasing of pesticides concentrations and exposure periods was detected in this experiment. The treatment with pesticides caused oxidative stress which led to increase in hepatic enzymes levels as it had destroyed the hepatocytes cell membrane and hepatic enzymes outflow to the blood [24].

A hepatic responses study of fish against water pollution found that the enzymes and biochemical parameters had either increased or decreased in the fish liver samples compared with control group which indicated the adverse effects of water pollution on the health of fish [25].

Table 4: Effect of groups and period in AST.

Mean ± SE of AST (IU/L)			
Group	**3 Weeks**	**6 Weeks**	**LSD value**
C	28.16 ±3.77 D a	28.17 ±3.77 D a	14.83 NS
G1	31.35 ±1.35 D b	38.80 ±0.91 CD a	7.01 *
G2	61.45 ±6.25 AB a	63.45 ±5.35 AB a	35.39 NS
G3	36.30 ±1.20 CD a	39.70 ±0.30 CD a	5.32 NS
A1	55.90 ±2.40 AB b	75.89 ±5.56 A a	18.99 *
A2	60.90 ±6.40 AB a	73.12 ±5.19 A a	35.47 NS
A3	69.10 ±4.50 A a	72.43 ±2.00 A a	21.18 NS
M1	45.05 ±7.95 BCD b	56.55 ±7.53 BC a	10.66 *
M2	49.70 ±12.20- B b	60.21 ±3.54 AB a	10.05 *
M3	44.06 ±3.41 BCD a	69.52 ±13.65 A a	60.16 NS
LSD value	2.068 *	18.16 *	---

AST mean value with different big-letters in the same column and small-letters in the same row are significantly different. * ($P \leq 0.05$).

Histological Changes in the Liver

The histological changed in the liver after 3 and 6 weeks exposure to different concentrations (1/10, 1/100 and 1/200 mg/L) of Goldate, Alexander and their mixture. Histological alterations observed in the liver tissues are summarized in Table 5.

Table 5: The histological changes in liver tissues of common carp after chronic exposure to Goldate, Alxander and their mixture.

	Exposure Period	
Group	**3 Weeks**	**6 Weeks**
C	normal appearance of hepatocytes & pancreatic acini.	
G1	show normal appearance of hepatocytes.	show sever jaundice associated with diffused hepatic degeneration, necrosis, exaggeration, and aggregations of bile pigments.
G2	show normal appearance of hepatocytes & pancreatic acini.	normal appearance of hepatocytes, pancreatic acini & mild congestion of sinusoidal vessels.
G3	normal appearance of hepatocytes, pancreas.	normal appearance of hepatocytes, pancreatic acini & blood vessel.
A1	mild vascular degeneration of hepatocytes and few necrotic hepatocytes.	mild vascular degeneration of hepatocytes, few necrotic hepatocytes, mild cellular swelling of few hepatocytes with vascular degeneration of pancreatic acini.
A2	normal appearance of hepatocytes, pancreatic acini & sinusoidal blood vessel.	normal appearance of hepatocytes and pancreatic acini & central sinusoid.
A3	normal appearance of hepatocytes and pancreatic acini.	normal appearance of hepatocytes, pancreatic acini & vessel.
M1	sever jaundice associated with diffused hepatic cellular swelling and aggregation of bile pigments.	sever jaundice associated with diffused hepatic cellular swelling and aggregation of bile pigments.
M2	show mild cellular swelling of few hepatocytes, moderate vascular degeneration and necrosis hepatocytes and vascular degeneration of pancreatic acini.	show moderate vascular degeneration and necrosis hepatocytes and vascular degeneration of pancreatic acini, mild cellular swelling of few hepatocytes.
M3	normal appearance of hepatocytes and pancreatic acini.	normal appearance of hepatocytes and pancreatic acini & central sinusoid.

The liver is the primary organ responsible for detoxifying xenobiotics. When fish are exposed to pesticides, significant structural changes occur within it. [26]. The liver is particularly susceptible to pollutants due to its extensive blood supply and role in metabolism [27].

In the control group, liver cells and hepatocytes were normal and arranged systematically which had a nucleus in the center. Fish liver tissues exposure to pesticides groups G1, A1, M1 and M2 showed different degrees of jaundice, cellular swelling, necrosis hepatocytes and vascular degeneration of pancreatic acini and aggregation of bile pigments.

Liver dysfunction might come from xenobiotics like pesticides that cause structural damage to hepatocytes. The treated fish liver contained moderately vacuolated hepatocytes and signs of necrosis in some liver cells which were likely brought on by the fish having to work too much to clear their bodies of toxins during the liver's detoxification process. Histological alteration registered progressive damages in hepato-renal tissues in treated fish with the increased exposure time compared to the control [28].

Fish liver tissue damaged comparatively more than other tissues perhaps as liver is the organ responsible for metabolic toxin detoxification nd its higher capacity to absorb pesticide residues [29].

Furthermore, like any toxicants, pesticides also stimulate a release of free radicals which destroy the vital macromolecules of the cells. So, the changes of hepatocytes are the only signs of the adverse impact of pollutants [30][31].

There were two reasons for swelling cell: it can occur directly owing to the denaturation of volume regulating ATPases, or indirectly it can happen when the cellular energy transfer routes essential to ionic regulation are disrupted [32].

The alterations in fish liver tissues observed may be due high exposure to pesticides which accumulate in hepatocytes causing larger inhibition of liver enzymes activities related to other affected tissues. Some fish species are mildly toxic to pesticides while other species are highly toxic. Therefore, the degree of damage in the tissues of pesticide-exposed fish may vary from fish to fish due to their varying physiological characteristics [33].

Same results were obtained in finding in the study of [34] that observed hyperplasia, vacuolation, disrupted hepatocytes, disintegrated blood vessels, disorganized hepatic canaliculi and focal coagulative necrosis in fish exposed to pesticides.

The risk of combined exposures of multiple pesticides has become an ongoing challenge for environmental and human health research in recent years. At environmentally relevant low concentrations, mixtures of pesticides exhibited additive toxic effects even if the individual compounds were not structurally similar [35].

Thus, Guo *et al.* [36] found that exposure to oxyfluorfen caused liver alteration in larvae of zebrafish as the residual Oxyfluorfen in the water has liver toxicity to water organisms. Also, that oxyfluorfen has toxic effects on hepatocyte with increasing concentration.

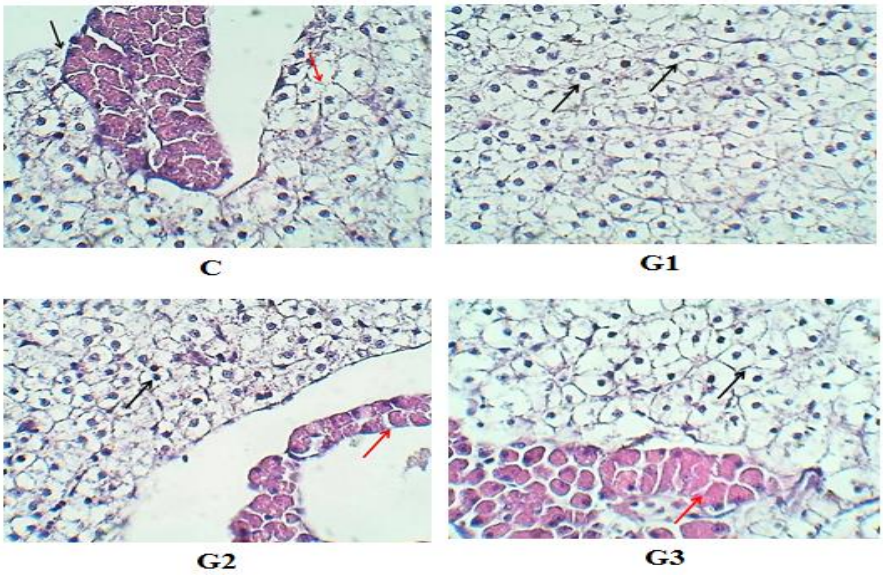

Figure 1: Section of liver after 3 weeks Goldate exposure.

C: Shows normal appearance of hepatocytes (Red arrow),& pancreatic acini (Black arrow). H&E stain.400x.
G1: Liver section treated with 1/10 mg/L for 3 weeks show normal appearance of hepatocytes (Black arrows). H&E stain.400x.
G2: Liver section treated with 1/100 mg/L for 3 weeks show normal appearance of hepatocytes (Black arrows) & pancreatic acini (Red arrow). H&E stain.400x.
G3: Liver section treated with 1/200 mg/L for 3 weeks show normal appearance of hepatocytes (Black arrow) & pancreas (Red arrow). H&E stain.400x.

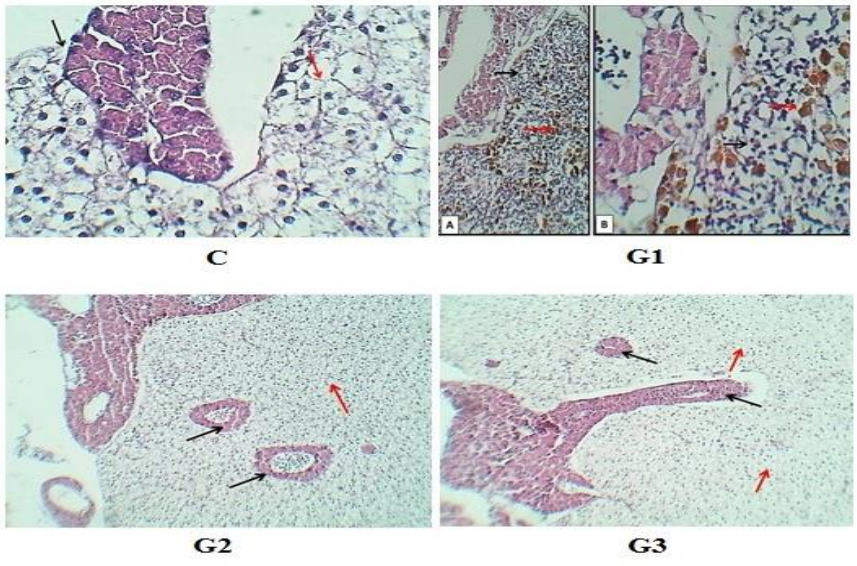

Figure 2: Section of liver after 6 weeks Goldate exposure.

C: Shows normal appearance of hepatocytes (Red arrow) & pancreatic acini (Black arrow). H&E stain.400x.
G1: Liver section treated with 1/10 mg/L for 6 weeks show sever jaundice associated with diffused hepatic degeneration & necrosis (Black arrows) & exaggeration with bile pigments (Red arrow). H&E stain.(A) 100x & (B) 400x.
G2: Liver section treated with 1/100 mg/L for 6 weeks show normal appearance of hepatocytes (Black arrows), pancreatic acini (Red arrow) & mild congestion of sinusoidal vessels H&E stain. 100x.
G3: Liver section treated with 1/200 mg/L for 6 weeks show normal appearance of hepatocytes (Red arrow), pancreatic acini (Black arrow). H&E stain.100x.

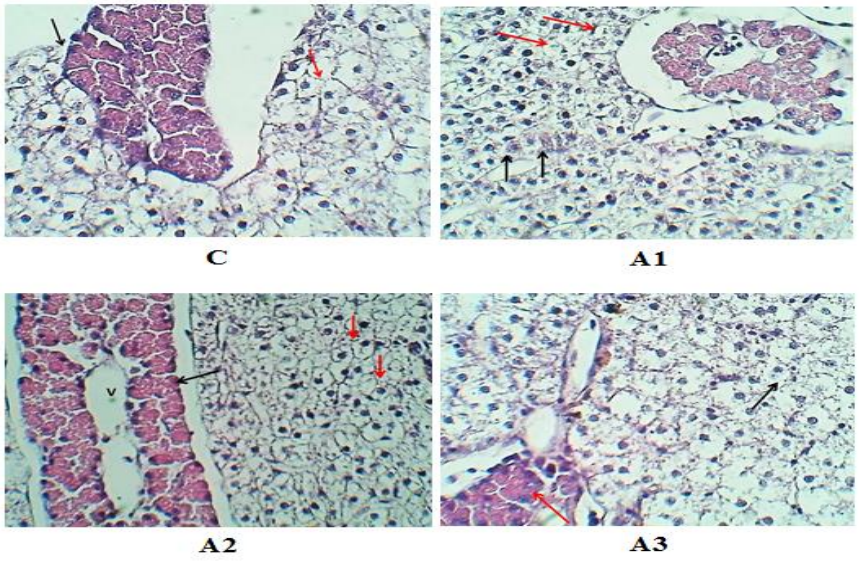

Figure 3: Section of liver after 3 weeks Alexander exposure.

C: Shows normal appearance of hepatocytes (Red arrow),& pancreatic acini (Black arrow). H&E stain.400x.
A1: Liver section treated with 1/10 mg/L for 3 weeks showing mild vascular degeneration of hepatocytes (Black arrow) & few necrotic hepatocytes (Red arrow). H&E stain.400x.

A2: Liver section treated with 1/100 mg/L for 3 weeks showing normal appearance of hepatocytes (Red arrows), pancreatic acini (Black arrow) & sinusoidal blood vessel (V) H&E stain.400x.
A3: Liver section treated with 1/200 mg/L for 3 weeks showing normal appearance of hepatocytes (Black arrows) and pancreatic acini (Red arrow) H&E stain.400x.

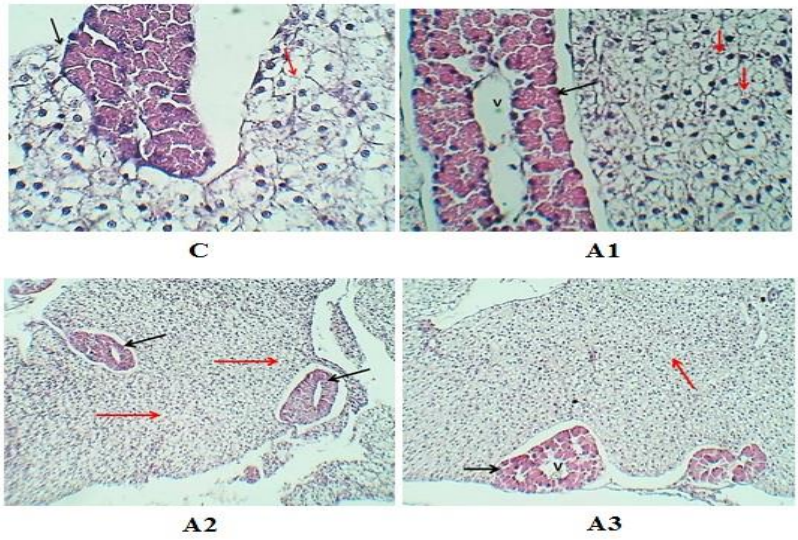

Figure 4: Section of liver after 6 weeks Alexsander exposure

C: Shows normal appearance of hepatocytes (Red arrow) & pancreatic acini (Black arrow). H&E stain.400x.
A1: Liver section treated with 1/10 mg/L for 6 weeks showing normal appearance of hepatocytes (Red arrows), pancreatic acini (Black arrow) & sinusoidal blood vessel (V) H&E stain.400x.
A2: Liver section treated with 1/100 mg/L for 6 weeks showing normal appearance of hepatocytes (Red arrow) & pancreatic acini (Black arrow). H&E stain.100x.
A3: Liver section treated with 1/200 mg/L for 6 weeks showing normal appearance of hepatocytes (Red arrow), pancreatic acini (Black arrow) & vessel (V) H&E stain. 400x.

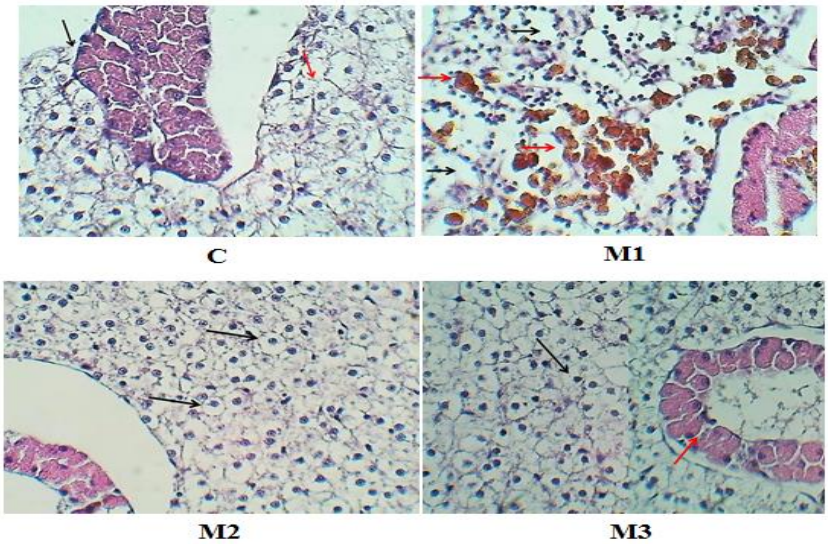

Figure 5: Section of liver after 3 weeks Mixture (Goldate + Alexander) exposure.

C: Shows normal appearance of hepatocytes (Red arrow) & pancreatic acini (Black arrow). H&E stain.400x.
M1: Liver section treated with 1/10 mg/L for 3 weeks shows sever jaundice associated with diffused hepatic cellular swelling (Black arrows) & aggregation of bile pigments (Red arrow). H&E stain. 400x.
M2: Liver section treated with 1/100 mg/L for 3 weeks shows mild cellular swelling of few hepatocytes (Black arrows) with normal appearance of pancreatic acini. H&E stain.400x.
M3: Liver section treated with 1/200 mg/L for 3 weeks showing normal appearance of hepatocytes (Black arrow) and pancreatic acini (Red arrow). H&E stain.400x.

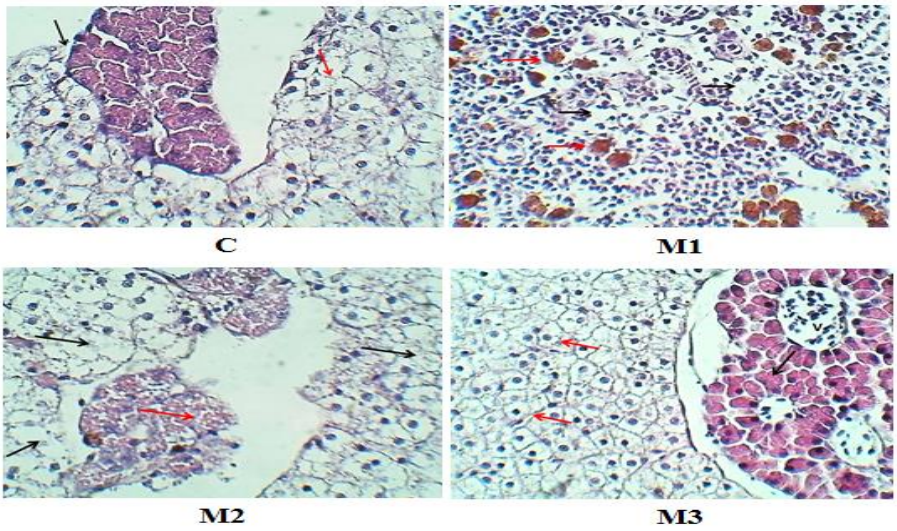

Figure 6: Section of liver after 6 weeks Mixture (Goldate + Alexander) exposure.

C: Shows normal appearance of hepatocytes (Red arrow) & pancreatic acini (Black arrow). H&E stain.400x.
M1: Liver section treated with 1/10 mg/L for 6 weeks shows sever jaundice associated with diffused hepatic cellular swelling (Black arrows) & aggregations of bile with bile pigments (Red arrow). H&E stain. 400x.
M2: Liver section treated with 1/100 mg/L for 6 weeks shows moderate vascular degeneration and necrosis hepatocytes (Black arrows) and vascular degeneration of pancreatic acini (Red arrow) H&E stain.400x**.**
M3: Liver section treated with 1/200 mg/L for 6 weeks shows normal appearance of hepatocytes (Red arrows), pancreatic acini (Black arrow) & central sinusoid (V). H&E stain.400x.

Conclusion

This experiment recorded a significant increase in the mean values of ALT and AST compared with control and significant differences in the ALT and AST mean values in the mixture of pesticides at M2 and M3 (lower concentration) reversed both Goldate and Alexander's effects that showed significant differences in groups G1 and A1 (high concentration) when comparing the differences of exposure times between 3 and 6 weeks.

The liver tissues of the fish exposure to pesticides groups G1, A1, M1 and M2 showed different effects degrees. These effects increased with increase in pesticides concentrations and exposure time. Also, these effects increased with the exposure to more than one pesticide at the same time. It can therefore be concluded that the mixture of Goldate + Alexander had more effects than each one alone.

Ethical Approval

This experimental and procedures involving fish were conducted in accordance with the animal care guidelines of University of Baghdad, Department of Biology, Baghdad, Iraq.

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