Egypt. J. Chem. Environ. Health, 1 (1):331-348 (2015)

Influence of vitamin C on cytogencity and biochemical parameters in chlorpyrifos intoxicated rats

Naglaa F. Al-Shap*; Sahar M. El-Sheshtawy*; Hanan M. Sobhy and Khaled E. El-Ekhnawy****

*Toxicology, Animal Health Research Institute, Tanta Branch &**Biochem. Toxicology. &Feed deff. Dep. Animal Health Research Institute, Dokki

Abstract

This study is aimed to evaluate the effect of vitamin C on Chlorpyrifos induced cytogencity, some serum biochemical parameters and liver pathology in rats. A total of twenty albino rats weighing (180g -200g) were used for this study. Rats were divided into 4 groups each of 5 rats. First group was given Chlorpyrifos 12 mg/kg bw. Second group was given Vitamin C 200 mg/kg bw + Chlorpyrifos 12 mg/kg bw. Third group was given vitamin C only and fourth group was left as control group. All doses were orally administered daily for 14 successive days. At the end of the experimental period, blood samples were collected from each rat for biochemical analysis. Rats were humanely euthanized and femur from all rats was taken for cytogenicity and liver was collected for histopathological study. Oral administration of chlorpyrifos for 14 days in a dose of 12 mg/kg b .w, significantly increased the percentage of micronucleated polychromatic erythrocytes (MPCEs), ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) .On other hand the administration of tested pesticide with vitamin c, reduce the frequencies of MPCEs and directed toward normal. The PCE/NCE ratio is a measure for red blood cell proliferation which gave a sign of toxicity or damage of some organs of the body. The effect on the liver is one of the main toxic effects of this product as there was a significant decrease in the total protein and albumin synthesized by the liver and increase in the leakage enzymes namely AST , ALT plus increased levels of in cholesterol and triglyceride. Histopathology revealed vacuolar of hepatocytes with random hepatocyte necrosis and mononuclear cell infiltration. Administration of vitamin C improved serum biochemical parameters and alleviated degenerative necrotic damage in the sections of liver examined histologically. In conclusion vitamin C has beneficial effects as it tends to dampen chlorpyrifos toxicity in rats

Introduction

Chlorpyrifos (CPF) has been one of the most widely used organophosphorus insecticides in agriculture and public health. Although some of its domestic uses have

been banned by United States Environmental Protection Agency **(US EPA 2000),** there is ample evidence that CPF remains one of the most common environmental contaminant (**Ambal et al., 2011).**

The wide application of CPF in public health and agricultural programs was accompanied by potentially hazardous impact on human, animals, plants and environment (water, air, soil and food) and causes severe acute and chronic poisoning (**Abdel-Tawab et al.,2011).**

CPF, like other organophosphate compounds is known to produce toxic effects through the inhibition of acetyl cholinesterase (AChE) activity which leads to accumulation of acetylcholine in the cholinergic receptors. It also induces oxidative stress leading to generation of free radicals which play an important role in DNA damage, lipid per oxidation and protein oxidation. **(Canadas, et al., 2005).**

In fact, the toxicity of CFP results in negative effects on many organs and systems such as the liver, kidney, nervous system, immune system and reproductive system **(Mansour and Mossa, 2011**).

Independent studies have found chlorpyrifos to be mutagenic or genotoxic in human, rat, mouse, Chinese hamster, toad, fish, fruitfly, and plant cells. California EPA stated it may be genotoxic **(Miller. 2004**).

Shalaby., (2013) was found that chlorpyrifos caused embryotoxic and teratogenic effects with growth retardation of fetuses.

Mansur et al .,(2013) stated that the oral intubation of chlopyrifos in dose 12mg/kg body weight for 14 day of treatment induce DNA damage and genotoxicity in male rats.

Vitamin C, a water-soluble vitamin, participates in a large number of cell functions, in addition to the antioxidant protective role in cell injury **(Blasiak and Kowalik, 2001).**

Vitamin C that has shown tremendous promise in attenuating organophosphorus-induced hematological, biochemical, and histopathological alterations, is hydrophilic and a very important free-radical scavenger in extracellular fluids, trapping radicals in the aqueous phase and protecting bio membranes from per oxidative damage (**Harapanhalli et al., 1996**).

The anticarcinogenic and antimutagenic roles of vitamin C have been tested in a variety of *in vivo* and *in vitro* systems exposed to radiation and pesticides **(Durak et al., 2009**). It prevents the increased production of free radicals induced by oxidative damage to lipids and lipoproteins in various cellular compartments and tissues **(Sies et al., 1992**).

Vitamin C accelerates the degradation of intra and extracellular proteins targeted to the lysosomal lumen by autophagic and heterophagic pathways, relevant for the removal of abnormal proteins that accumulate with aging **(Martin *et al*., 2002**).

Therefore, the present study was designed to evaluate the ameliorative effect of vitamin C in attenuating the deficit in liver enzymes (AST and ALT), some serum biochemical parameters (total protein, albumin, globulin, cholesterol and triglyceride), and genotoxicity and liver histopathology in rats intoxicated by chlorpyrifos (CPF).

Materials and Methods

Chemicals and reagent:

Chlorpyrifos (Pestban® 48% EC) was obtained from Agrochem, Alwatneia Co., Alex., Egypt; animals were treated once daily with 12 mg/kg body weight (**Mansur et al., 2013**) that is subleathal dose and is equivalent to 1/17, according to the LD_{50} of CPF, since the oral LD_{50} of CPF in rats was evaluated as 200 mg/kg BW (**Savithri et al., 2010).**

Vitamin C (Ascorbic acid) from Unipharma Company, Egypt, evaluated for its safety effects and a dose of 200 mg /Kg body weight was selected according to **(Aly et al., 2010).**

Liver dysfunction biomarkers: All serum biomarkers were determined using a commercial kit in accordance with manufacturers' instructions. The activity of cellular enzymes such as aspartate aminotransferase and alkaline phosphatase (**Reitman and Frankel, 1957**) alanine transaminase (**Sherwin, 1996**) were determined in sera. While, the concentration of albumin and total protein were determined by the methods of **Ellman et al. (1961), Westgard and Poquette (1972)** and **Gornall et al. (1949),** respectively. Calculation of globulin in serum: Serum proteins comprise of albumin and globulin (alpha, beta and gamma). Therefore, concentration of globulins can be calculated as follows:

Globulins (g dL-1) = total protein (g dL-1) -albumin (g dL-1) and thus Albumin/Globulin ratio (A/G) could be estimated

Total cholesterol level (TC) was assayed according to (**Flegg, 1973 and Allain et al., 1974)** using spinreact reagent Kits; Spain. Triglycerides (TG) was determined by the method of **(wahlefeld, 1974)** using spinreact reagent Kits; Spain.

Spectrophotometric measurements:

The Spectrophotometric measurements were performed by using a Shimadzu UV-VIS Recording 2401 PC (Japan).

Animals and groups:

20 Male rats of the Wistar strain (*Rattus norvegicus*) weighing 180-200 g were obtained from a private Animal Breeding House for lab animal. Animals were kept in

clean plastic cages with free access to food (standard pellet diet) and tap water *ad-libitum*, under standardized housing conditions in the laboratory animal room. After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to four groups, each consisting of five rats, as follows: First group (CPF) treated by oral intubation of chlorpyrifos only (12 mg/kg), second group(VC) treated by oral intubation of vitamin C (200 mg/kg) only. Third group (CPF+VC) were pretreated by oral intubation with vitamins C (200 mg/kg) followed by CPF (12mg/kg), 30 minutes later and fourth group (C) control received food and water only.

All CPF and VC were dissolved in distilled water and given via oral route for 14 consecutive days. Dosages of each administered were daily freshly prepared and adjusted weekly for body weight changes. The control group received an equivalent volume of distilled water (0.5 mL/rat).

The experimental protocols and procedures were approved by the Local Ethics Committee at the National Research Centre (NRC), Dokki, Cairo, Egypt.

Blood samples:

In all groups, body weights were recorded at the beginning of experiment and after 7 for adjustment the dose, the blood samples were drawn from all rats under ether anesthesia by puncturing the reteroorbital venous plexus of the animals with a fine sterilized glass capillary and collected in glass tubes to separate the sera. Within 20 min of blood collection, the sera samples were drawn from blood after centrifugation at 3500 rpm for 10 min at 4°C, using Hereaeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany. The sera was kept in a deep freezer (-20°C) until analyzed.

After blood collection, the rats were scarified by cervical dislocation, femur bone were taken for cytogenic examination. Liver of rats was quickly removed then; the liver samples were collected preserved in 10% formal saline solution for histopathological examination.

Cytogenic examination:

In order to assess the possible mutagenic effect of chlorpyrifos, the micronucleus test was performed to detect chromosomal associated with the treatment Micronuclei were identified as dark – blue – staining bodies in the cytoplasm of the polychromatic erythrocytes (PCEs).

Following the protocol established by **salamon etal., (1980),** bone marrow cells of rats were extruded with a pin into clean dry glass lid, homogenized with two drops of fetal calf serum , cells were smeared on the slide , homogenized erythrocytes (PCEs/1000 animal) were screened for micronuclei , and the changes in mitotic activity **(Harts and engberg – Pederson ,1983 and Al-Bekeairi et al ,1991)** were assessed on the basis of the ratio (polychromatic –normochromatic erythrocytes – PCE-NCE ratio) .

Histopathological studies:

Liver samples were dissected and fixed in formalin, dehydrated and imbedded in paraffin wax. Then, the sections were stained by haematoxylin and eosin (H and E). Two slides were prepared for each rat, each slide contained two sections and examined for histopathological changes under light microscope.

Statistical Analysis:

The results were subsequently analyzed following the statistical method established by **snedecor and Cochran (1973**) in order to determine whether a dose group was positive or negative.

Results and Discussion

Cytogenicity:

Table(1): displays the frequencies and the percentage of micronucleated polychromatic erythrocytes (MPCEs) and the ratio of PCE/NCE in control animals and the groups of rats treated with chlorpyrifos alone or combined with V.C. Chlorpyrifos caused increased percentage of MPCEs when compared with other groups. The increased percentage of MPCE, were statistically significant at p level 0.001. Our results agree with that of **(Manjula et al., 2011).**

Chorpyrifos is an alkylating agent that chemically altered polynucleated chains. This essentially inhibits DNA synthesis including one or more type of chromosomal aberration in the cells, furthermore its toxicity may be attributed in part by generation of reaction oxygen species (**bender et al , 1974, saul-sbury et al 2009 and mansour et al 2009).** Dose dependent increase in frequency of MNPCE was reported by **(Salah et al, 1998**). The PCE/NCE ratio decreased significantly toward the control value when rats treated with chlorpyrifos combined with Vit.C.

Administration of Vit C and chlorpyrifos reduce the micronucleus frequencies in polychromatic erythrocytes and directed toward control. Vitamin C affects oxidative changes which occur in cell organelles **(Tanake et al, 1997)** thus protect DNA from damage, reduce the chromosomal aberrations and micronuclei formation caused by force radicals **(Kevin et al 1999, yuko et al 2003).**

The studies conducted with rodent and insect cell lines suggest that chlorpyrifos is genotoxic **(Amer and Fahmy 1982; Patnaik and Tripathy 1992; Sobti et al. 1982**; **Woodruff et al. 1983**). A dose-response effect of chlorpyrifos on the induction of micronuclei in bone marrow has been observed **(Amer and Fahmy 1982).**

Liver enzyme:

The liver, the key organ involved in numerous metabolic functions and plays a central role in the detoxification process and faces the threat of maximum exposure to xenobiotic and their metabolic by-products **(Meyer and Kulkarni, 2001).** Serum enzymes including ALT& AST are mainly used in the evaluation of hepatic damage.

As show in Table 2, our results revealed that there was a significant ($P<0.05$) increase in AST and ALT activity in the CPF group compared to either C or VC group. Supplementation of V.C was mitigating the adverse effects of exposure to CPF toward control levels, but the increase in the enzyme activates were still significantly increased ($p\leq0.05$) compared to the control or VC group. This fact is a conventional indicator of liver injury (**Rao, 2006).** Elevation of serum AST and ALT indicates liver damage **(Akhtar et al., 2009**) When the liver cell membrane is damaged, varieties of enzymes normally located in the cytosol are released into the blood stream. . This agrees with the low ALT and AST activities in the liver of rats exposed to CPF in the previous findings of (**Zama et al., 2007**). Therefore, the increase in these enzymes may be due to liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane takes place **(Meyer and Kulkarni, 2001; Khan et al., 2005).**

Administration of the antioxidant vitamins C exerted protective effects on AST & ALT levels in co-exposed CPF rats. The protective effects of V.C have been reported previously by other investigators **(Khan and Sinha, 1994; Gultekin et al., 2001; Hong et al., 2002; Aly et al., 2010).**

Lipid profile:

The results in table (2) shows that the treatment of rats with CPF induced significant ($p < 0.05$) increases in the serum total cholesterol and triglycerides compared with C and VC group. . Although, serum total cholesterol and triglycerides concentration in the (VC+CPF) group decreased compared to that recorded in the CPF group, but it was still high compared to either C or VC group.

The same trend was seen with other organophosphorus pesticide (**Kalender et al., 2005).** Previous studies demonstrated increases in the concentration of serum triglycerides in the experimental animals that were treated with different insecticides, including the organophosphate, dichlorvos **(Ranjbar et al. 2002**) and diazinon **(Ibrahim and El-Gamal, 2003**). This elevation of serum or plasma triglycerides and total cholesterol has been attributed to an inhibition of the lipase enzyme activity of both the hepatic triglycerides and plasma lipoproteins (Goldberg et al., 1982).this result also agree with **(Newairy and Abdou,2013). (Ambal et al., 2011**) had shown the ability of vitamin C to mitigate toxicity induced by CPF.

Serum protein:

As show in Table (3), CPF significantly ($p \leq 0.05$) reduced serum total protein in comparison to controls. Combination therapy with V.C significantly ($p \leq 0.05$) restored CPF effect to within the normal limits. Although, the total protein concentration in the (VC+CPF) group increased compared to that recorded in the CPF group. There was decrease in the concentration in the (VC+CPF) group compared to either C or VC group.

The albumin concentration in the CPF group decreased significantly ($P<0.05$) compared to that recorded in the C or VC group. Although, the albumin concentration in the (VC+CPF) group increased compared to that recorded in the CFP group. There was decrease in the albumin concentration in the (VC+CPF) group compared to either C or VC group.

The globulin concentration in the CPF group were significantly ($P<0.05$) decreased compared to VC or C group, combination of vitamin C with CFP improve globulin level toward normal but still decreases compared to VC or C group.

The effect of treatments on albumin/globulin ratio is shown in Table 3. The albumin/globulin ratio in the CPF group increased when compared to either C or VC+CFP group. Giving vitamin C improve levels of total protein, albumin and globulin toward normal levels.

Hypoglobulinemia recorded following CPF exposure has been partly attributed lymphocytic leukopenia **(Ambali, 2007), (Ambli et al., 2010d), (Goel et al., 2006)**. Apoptotic damage to the immune cells has also been described following pesticides exposure **(Rabideau, 2001).** The decrease in total protein in CPF treated group may be due to liver dysfunctions and disturbances in the biosynthesis of protein. Previous studies showed that protein content was changed in human (**Nabila et al., 1990**), rats **(Enan et al., 1982;El-Bakary,1993; Mansour and Mossa,2010a,b,2011)** and (**Khan and Tabassum, 2003**) as a result of insecticide exposure.

These results agree with that reports of **(Peeples et al., 2009).** Normally, the reduction of serum protein and albumin levels as sown I Table 3 indicates a liver disease. This reduction could be attributed to the changes in the metabolism and the synthesis of protein and free amino acids in the liver **(Li et al., 2007).** They suggested that albumin could be used as a biomarker of CPF-toxicity.

Pretreatment with vitamin C has been shown by the present study to reduce hypoproteinemia, hypoalbuminemia and the relative hypoglobulinemia associated with exposure to CPF. The reduction of the hypoalbuminemia level by vitamin C may be due

to protection of the liver from oxidative damage due to exposure to CPF, due to its antioxidant effect. The improved globulin concentration by pretreatment with vitamin C may also be due to reduction in apoptotic damage to the WBCs due to its antioxidant properties. Vitamin C has been shown to mitigate leukopenia induced by CPF poisoning **(Ambali SF 2009).**

Histopathology:

Histopathological results of the current study demonstrated that 14-day exposure of rats to chlorpyrifos 12 mg kg-1 b.w. Fig (1) Liver showed multiple necrotic foci which replaced by mononuclear inflammatory cell infiltration. Fig (2) showed most central veins and hepatic sinusoids were dilated and filled with blood. Activation of kupffer cells could be detected. Fig (3) showed most hepatocytes revealed vacuolar degeneration with pyknotic nuclei. Multiple degenerative changes were detected in hepatocyte, oedema in portal area, dilatation and congestion in the central veins of the liver. Previous work has reported dilatation of central vein, degradation, congestion, oedema, hyalinosis, fibrosis and necrosis in the liver of rats (**Mansour and Mossa, 2010a, 2011).** Also, hepatocellular degeneration and necrosis was recorded in rat treated with profenofos (**Mansour et al., 2008)** Malathion and diazinon.

The hepatic function tests corroborated the histopathological lesions observed in the present study. Degeneration and necrosis in the hepatocytes, inflammatory cells infiltration, and Kupffer cells proliferation were frequently observed in CPF-treated group. These observations indicated marker changes in the overall histoarchitecture of liver in response to CPF. Our results are supported by other studies conducted on CPF and other OP insecticides (**Heikal et al., 2011), (Tuzmen et al., 2008), (Mansur and Mossa 2010), (Mansur 2011**), the co-treatment of vitamin C improved the histological alterations induced by CPF, which could be attributed to the antiradical and antioxidant of this vitamin. Moreover, these results are in good accordance with those obtained by other studies which have postulated the beneficial role of vitamin C on histopathological and enzymatic changes of rats **(Combs and Combs 1984), (McPherson 1994),(Ozardalıa et al.,2004**) .

Conclusion:

In conclusion, the results of the present study suggest a protective effect of ascorbic acid against administration of CPF which causes alteration in biochemical parameters indicating damages to some organs such as the liver. Pretreatment with vitamin C restore the biochemical alterations caused by administration of CPF.in conclusion this study has demonstrated that the antioxidant vitamin C has an ameliorative effect on clinical, hematological and serum biological parameters altered by prolonged administration of CPF. In addition to its antioxidant activity vitamin C is

known to perform other actions that enhance its protective effect in organophosphorus induced toxicity.

Table 1: The incidence of M PCE and the relation of PCE /NCE in rats exposed to CPF for 14 days and the protective effect of vitamin C. (N =5).

Group	PCE	%	MPCE	NCE	PCE/NCE ratio
CPF	5000	1.56	15.6 ± 4.278***	952	4.82±1.74*
VC	5000	0.54	5.4±1.2	1.543	2.17±0.3
CPF+VC	5000	0.8	8.0±1.6	1820	1.28±0.24*
C	5000	0.56	5.6±2.61	2183	2.33±0.39

CPF:chlorpyrifos, VC: Vitamin C, C: control , PCE :polychromatic erythrocytes, MPCE: micronucleated polychromatic erythrocytes, NCE :normochromatic erythrocytes. Values are Means ±SD; n = 5; Statistical difference from the control: Significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$.

Table 2: ALT, AST activities, total cholesterol (TC) and triglyceride (TG) levels in the sera of rats exposed to CF for 14 days and the protective effect of vitamin C.

PARAMETERS	CPF	VC	CPF+VC	C
AST (UL^{-1})	57.8± 0.83**	33.5± 1.72	47.8± 0.91*	37.2± 1.22
ALT(UL^{-1})	61.6± 2.42**	38.7±0.73	50.3± 1.15*	40.8± 1.73
TC(UL^{-1})	132.5 ± 1.23*	78.6 ± 7.12	121.4± 0.84*	96.5 ± 5.31
TG $^{(UL-1)}$	118.8 ± 5.31**	69.7 ± 2.34	88.5± 0.96	75.2 ± 2.14

CPF: chlorpyrifos, VC: Vitamin C, C: control, AST: Aspartate transaminase, ALT: Alanine treansaminase, TC: total cholesterol, TG: Triglyceride, Values are Means ±SD; n =5 Statistical differences from the control: Significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$.

Table 3: Serum total protein, albumin, globulin concentration and A/G ratio in rats exposed to CPF for 14 day and the protective effect of vitamin C.

groups	CPF	VC	CPF+V	C
Total protein ($g\ dL^{-1}$)	6.17± 1.21*	7.86± 1.55	6.98± 1.31*	7.81± 0.91
Albumin (A) ($g\ dL^{-1}$)	3.24± 0.94*	3.72± 3.22	3.64± 0.48*	3.75± 0.83
Globuline (G) ($g\ dL^{-1}$)	2.93± 0.73**	4.14± 2.76	3.34± 0.43*	4.06± 0.33
A/G ratio ($g\ dL^{-1}$)	1.10± 1.41	0.89±1.33	1.08± 0.77	0.92± 0.13

CPF: chlorpyrifos, V.C: Vitamin C, Values are Means il±SD; n = 5; Statistical difference from the control: Significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$.

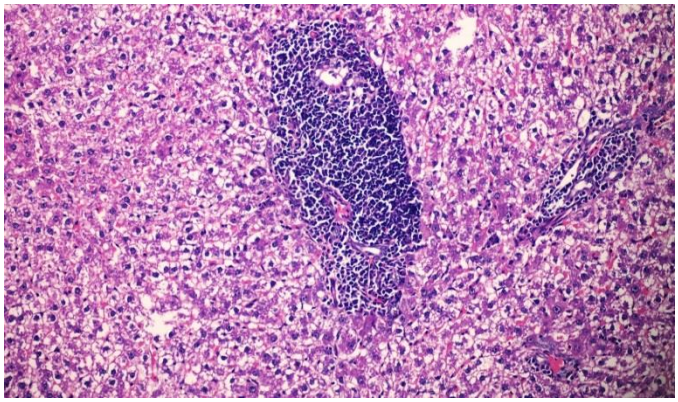

Fig 1:- Liver shows large focus of mononuclear cell infiltration around portal area H&E X 100

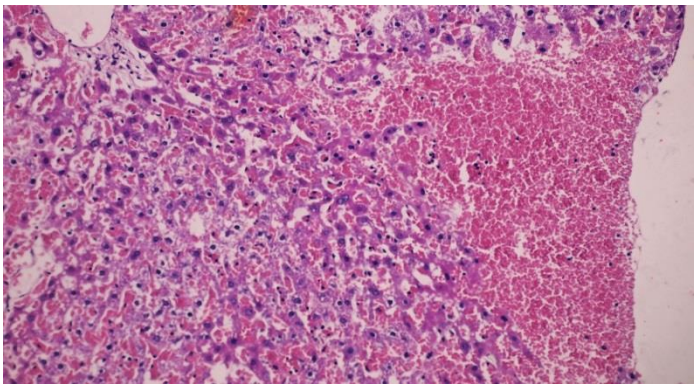

Fig 2:- Liver shows sup capsular hemorrhage and dilatation of hepatic sinusoids which filled with blood H&E X 200

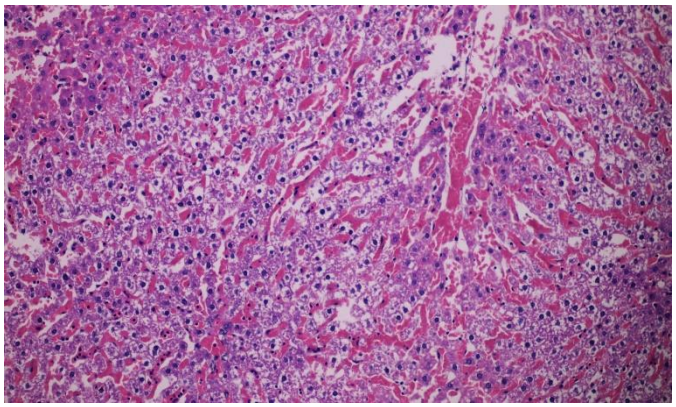

Fig 3:- Liver shows dilated central vein and hepatic sinusoids filled with blood. Most hepatocytes had vacuolar degeneration with pyknotic nuclei. H&E X 200

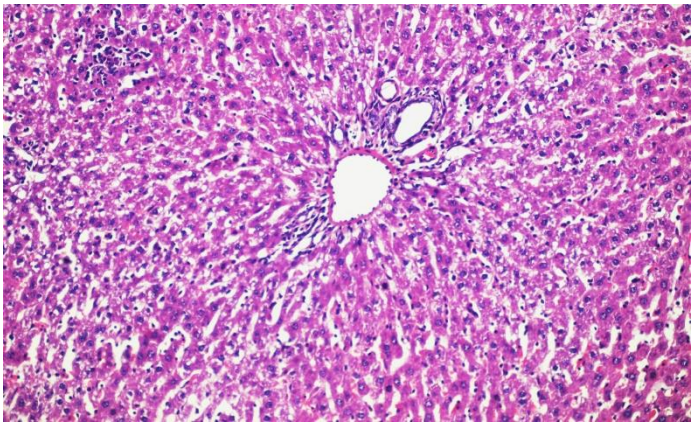

Fig 4 :- Liver of rats received vit C and chloropyrifos shows degenerative changes of hepatic cells with necrotic foci on the upper left side with mono nuclear cell infiltration H&E X 200

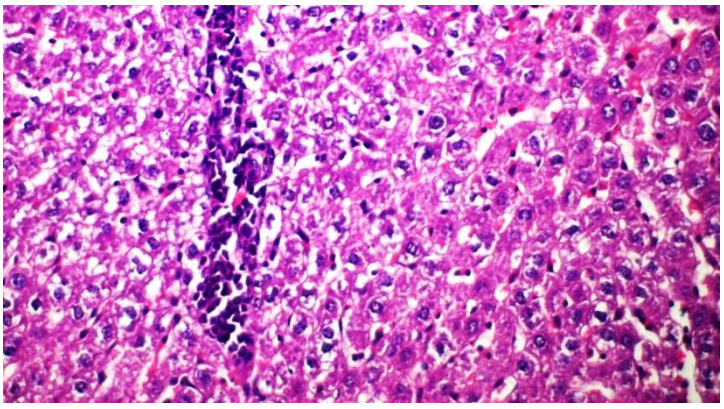

Fig 5:- Liver of rats received vit C and chloropyrifos shows mono nuclear cell infiltration mainly lymphocyte as well as hepatocytes had some vacuolar degeneration H&E X 400.

ACKNOWLEDGMENT:

Great thankful for Prof. Dr. Abass Aly Professor of Pathology, Animal Health Research Institute, Dokki for reading and interpreting the histopathological sections.

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