

## **Histopathological Effects of Doxorubicin and Nanoparticle Zinc oxide on DNA damage and Hepatotoxicity induced by CCl<sub>4</sub> in rats.**

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### **Abstract**

Nanoparticles are important scientific tools that have been recruited in various biotechnological, pharmacological applications. Zinc oxide nanoparticles are used considerably for its catalytic, electrical, optoelectronic and photochemical properties. Quinine- containing anthracycline antibiotic doxorubicin (DXR) has been used for the treatment of a wide variety of cancers, despite of its high antitumor efficacy DXR use in chemotherapy has been largely limited due to its cardiac, renal and hepatic toxicity. The present study has been carried out to evaluate the effect of Doxorubicin and ZnO NP when used together and separately against the CCl<sub>4</sub> induced hepatotoxicity in the rats. The damage of the liver caused by CCl<sub>4</sub> and Doxorubicin was evident by DNA gel electrophoresis and histopathology. In the present study, Forty eight white Albino rats (Sprague Dawley strain) were divided into 8 equal groups of 6 rats each for 28 successive days. Group one was kept as a control –ve, second and third groups were injected intraperitoneally with DOX(6 mg/kgb.wt) and nZnO(5 mg/kgb.wt) respectively for 3 successive days. While fourth group was injected intraperitoneally nZnO followed by DOX for the same period. On the other hand, other four groups were injected subcutaneously with CCl<sub>4</sub> 50% (0.1 ml/100g.b.wt.twice/week for two weeks )to induce DNA damage and Hepatotoxicity. One of this groups was kept as a control +ve (CCl<sub>4</sub> groups).Second and third groups were injected intraperitoneally with DOX (6 mg/kgb.wt) and nZnO (5 mg/kgb.wt) respectively for the same period.While fourth group was injected intraperitoneally nZnO followed by DOX for the same period. At the end of experimental period the liver and spleen tissue were used for histopathological assessment and illustrate DNA damage in hepatocytes. Vacuolar degeneration associated with proliferation of fibrous connective tissue surrounding the hepatic cells with dilated hepatic artery was observed in the group which received CCl<sub>4</sub> and doxorubicin. Examination of the spleen showed severe depletion in lymphoid elements in both white and red pulp especially in the center of lymphoid follicles. Subcutaneous injection of CCl<sub>4</sub> induced DNA fragmentation and intraperitoneal injection of doxorubicin showed DNA fragmentation while rats injected Doxorubicin and ZnO NP showed less DNA fragmentation. The ZnO NP treated animals showed reversal of toxic effects in the liver cells.

Thus, it can be concluded that the reduce DNA damage and improved histology of the liver and spleen as seen in the histopathological observation on animals treated with ZNO NP as compared to that seen in animals administered only CCl<sub>4</sub> or only Doxorubicin indicated possibility of the ZnO NP being able to induce accelerated regeneration of the liver and spleen.

### **Introduction**

Nanotechnology deals with nanoparticles that are atomic or molecular aggregates characterized by size less than 100 nm. These are actually modified form of basic elements derived by altering their atomic as well as molecular properties of elements(Wang, 2004 and Suche et al., 2006). Nanoparticles are important scientific tools that have been recruited in various biotechnological, pharmacological applications. The potential of nanomedicine has fueled the design and deployment of novel engineered nanoparticles (ENPs) for many biomedical applications. ENPs have high surface area-to-volume ratios and many characteristics providing selected electrical, magnetic, and structural properties that may prove useful in the diagnosis and treatment of diseases. Currently, various metal oxide ENPs are being explored as vehicles for cancer treatments (Brannon-Peppas and Blanchette, 2004), tumor detection(Yang et al., 2012 ), and gene-delivery therapies(Zhang, 2010). Of particular interest in nanomedicine is ZnO ENPs. Zinc oxide nanoparticles are used considerably for its catalytic, electrical, optoelectronic and photochemical properties (Brida et al., 2002; Wang, 2004; Ashour et al., 2006; Suche et al., 2006). ZnO nanoparticles are used in the cosmetic industry, typically in sunscreens and facial creams (Nohynek et al., 2007; Nohynek et al., 2008).

Quinine- containing anthracycline antibiotic doxorubicin (DXR) has been used for the treatment of a wide variety of cancers, despite of its high antitumor efficacy DXR use in chemotherapy has been largely limited due to its cardiac, renal and hepatic toxicity (P.K Single et al., 1999; E. Fadilliogiu, 2003 ). Doxorubicin has been used for the treatment of various malignancies including breast tumors, bile duct, endometrial tissues, esophagus, liver as well as bone tumors (Sule Ayla et al., 2011).

Liver is the most important metabolic organ in the body. It plays a major role in the metabolism, detoxification, storage and secretory functions in the body of animals (Swarnalatha and Reddy 2012). The basic structural unit of the liver is the hepatocytes, those cells are grouped in interconnected plates. Each classical liver lobule is formed of a polygonal mass of tissue in which the hepatocytes are radially disposed. Between those cells a sinusoidal liver capillaries and bile canaliculi are presents. At the center of each hepatic lobule there is a central vein. Portal areas are present at the periphery of each lobule and are occupied the portal triads which consist of a branch of the portal vein, a branch of the hepatic artery and a branch of the bile duct system(EL-Rakhawy, 2000; Ganqueria et al., 2003). Various

xenobiotics or oxidative stress can produce deleterious effects on these important functions of liver (**Kale et al., 2012**). Liver injury due to chemicals (or) infectious agents may lead to progressive liver fibrosis and ultimately cirrhosis and liver failure (**Anand, 1999**).

Carbon tetrachloride CCl<sub>4</sub> is widely used for experimental induction of liver damage. The principle causes of carbon tetrachloride (CCl<sub>4</sub>) is induced hepatic damage in lipid peroxidation and decreased activities of antioxidant enzymes and generation of free radicals (**Castro et al., 1974; Poli, 1993**).

The present study has been carried out to evaluate the effect of Doxorubicin and ZnO NP when used together and separately against the CCl<sub>4</sub> induced hepatotoxicity in the rats.

## Materials and Methods

### Chemicals

#### -Doxorubicin :

DOX (EbewePharma co. Austria) 2 mg/ml concentrate for solution for infusion (vial of 25 ml contains: 50 mg Doxorubicin HCl) The chemical name of doxorubicin HCl is (8S,10S)-10-[(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxohexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride. The molecular formula is C<sub>27</sub>-H<sub>29</sub>-NO<sub>11</sub>•HCl; its molecular weight is 579.99 (**PuranBadkoobeh et al., 2013**).

#### -Synthesis of Zinc oxide NPs :

0.02M aqueous Zinc acetate dihydrate was dissolved in 50 ml distilled. At room temperature, aqueous 2.0M NaOH was added drop by drop to reach pH 12. Which was then placed in a magnetic stirrer for 2hr. The white precipitate formed was washed thoroughly with distilled water followed by ethanol to remove the impurities. The precipitate was dried in a hot air oven for overnight at 60°C. Complete conversion of Zn (OH)<sub>2</sub> into ZnO NPs took place during drying.

The reactions involved in the synthesis process are as follows:



that the obtained products are composed of near flower shape morphology with the average size in the range of 0.5 $\mu$ m (**Gnanasangeetha and SaralaThambavani, 2013; Theodore, 2006**).

#### -Carbon tetrachloride ( CCl<sub>4</sub> ):

Carbon tetrachloride (99.9 purity) was purchased from Sigma chemical company. It was used as 50% in propylene glycol to rats at dose (0.1 ml /100g.b.wt) twice/week subcutaneously according to **Borah et al., (2004)**.

### **Animals**

Forty eight white Albino rats (Sprague Dawley strain) of an average body weight 130-165 g. They were obtained from the laboratory of colony, Ministry of Health and population, Helwan, Cairo, Egypt. Animals were acclimatized to laboratory condition before being used. Rats were fed on standard diet supplying the essential vitamins, trace elements and water supply was given ad libitum.

### **Experimental design**

This was planned to study the effects of Doxorubicin and Nanoparticle Zinc oxide in normal and Hepatotoxicity induced by CCl<sub>4</sub> in rats. For this purpose 48 adult rats were divided into 8 equal groups of 6 rats each for 28 successive days.

**Group 1** : Kept as control negative.

**Group 2** : Injected intraperitonily with DOX (6 mg/kgb.wt).

**Group 3** : Injected intraperitonily with nZnO (5 mg/kg/day) .

**Group 4** : Injected intraperitonily with nZnO (5 mg/kg/day) followed by DOX (6 mg/kgb.wt) one day before.

**Group 5** : Kept as control positive, it was injected subcutaneously by CCl<sub>4</sub> (0.1ml/100gb.wt.) twice/week for to weeks.

**Group 6 ( CCL4 group )** : Injected intraperitonily with DOX (6 mg/kgb.wt).

**Group 7 ( CCL4 group )** : Injected intraperitonily with nZnO (5 mg/kg/day) .

**Group 8 ( CCL4 group )** : Injected intraperitonily with nZnO (5 mg/kg/day) followed by DOX (6 mg/kgb.wt) one day before.

All groups were treated for 3 days.

### **Sampling:**

#### **Histopathological studies**

PM.examination of liver and spleen was done immediately after sacrificing the animals. A small portions were fixed in 10% neutral buffered formalin as described by **Luna (1968)**. Thin sections of 4-5  $\mu$ m were taken, stained with Haematoxylin and Eosin and histology was studied.

#### **DNA gel electrophoresis.**

The extent of DNA fragmentation (DNA ladder) has been assayed by electrophoresing genomic DNA samples, isolated from normal as well as experimental rat liver, on 1% agarose/ethyidium bromide gel by the procedure described by **Sellins and Cohen(1987)**. DNA extraction: Principle: High quality genomic DNA was extracted from (-80°C preserved liver samples of all treated and control groups) by precipitation of protein and other contaminants and further precipitation of high molecular weight genomic DNA by absolute ethanol(**Sambrook et al., 1989**).

## Results

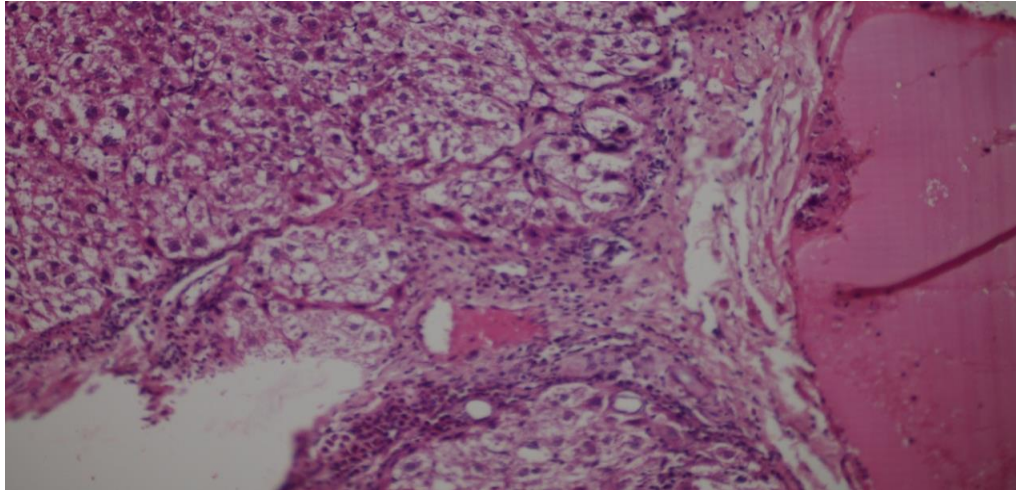
Histopathological studies of the liver section of control and experimental animals were shown in (fig.1-7), was carried out to test the effect of Doxorubicin and ZnO NP in hepatotoxicity induced by CCL4. In this study marked histological changes were observed in all groups of rats . Vacuolar degeneration associated with proliferation of fibrous connective tissue surrounding the hepatic cells with dilated hepatic artery was observed in the group which received CCl4. The CCl4 induced hepatotoxicity produced in rats leading to hepatic injury triggers the generation of toxic radicals. And vacuolar degeneration in some hepatic cells especially central vein was observed in the group which received Doxorubicin. Fig-1 shows the liver section of positive control animals( Group 5) which showing severe vacuolar degeneration associated with proliferation of fibrous connective tissue surrounding the hepatic cells with dilated hepatic artery which is congested. Fig-2 shows the liver section of (Group 2) showing vacuolar degeneration in some hepatic cells especially central vein. Fig-3 shows the liver section of Group 6 ( CCL4 group ) showing moderate vacuolar degeneration of hepatic cells. Fig-4 the liver section of Group 7 ( CCL4 group ) showing moderate vacuolar degeneration in hepatic cells and congestion in some blood vessels. Fig-5 the liver section of Group 7 ( CCL4 group ) showing moderate infiltration of mononuclear cells in portal area associated with mild dilatation of portal blood vessels.

The ZnO NP treated animals showed reversal of toxic effects in the liver cells. Fig-6 shows the liver section of( Group 4) Vacuolar degeneration in some hepatic cells is improved to mild degeneration. Fig-7 shows the liver section of Group 8 ( CCL4 group ) Accumulation of inflammation cells around portal area with congestion in portal veins.

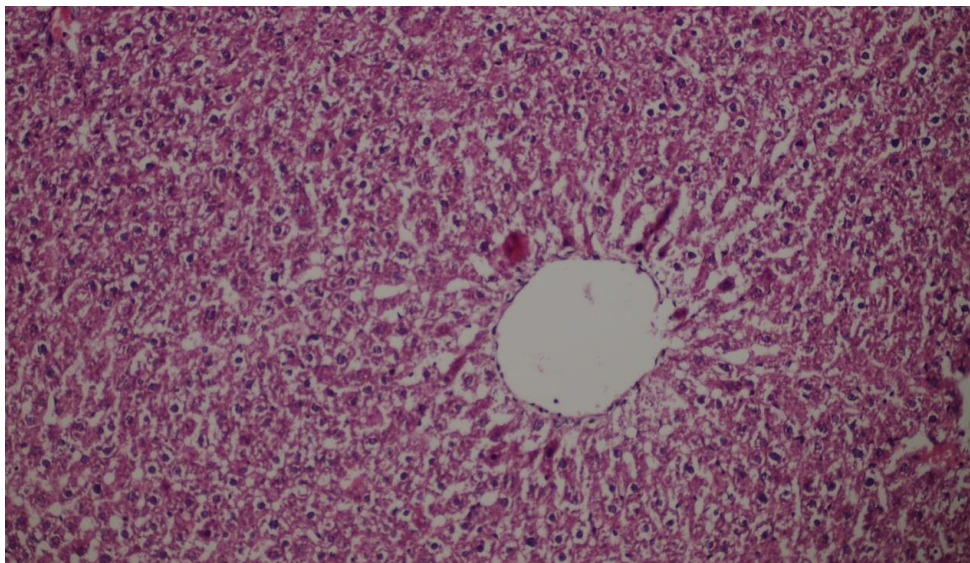
Histopathological studies of the spleen section of control and experimental animals were shown in (fig.8-14), Fig-8 (Group 5) showing severe depletion in lymphoid elements in both white and red pulb especially in the center of lymphoid follicles. Fig-9 (Group 2 ) revealing mild depletion in lymphocytes in both white and red pulb. Fig- 10 (Group 6, CCL4 group) revealing hyperplasia of lymphoblast in the center of some white pulb. Fig-11 (Group 6,CCL4 group )revealing moderate depletion in lymphocytes in white pulb. Fig-12( Group 3) revealing normal histological structure. The ZnO NP treated animals showed reversal of toxic effects in the spleen cells Fig- 13(Group 7 ( CCL4 group ) revealing marked improvement in pathological changes. Fig- 14 (Group 8 ( CCL4 group ) revealing marked improvement in pathological changes.

Subcutaneous injection of CCl4 induce DNA fragmentation and intraperitoneal injection of doxorubicin showed DNA fragmentation while rats injected Doxorubicin and ZnO NP showed less DNA fragmentation Figure (15,16 ).

The improved histology of the liver and spleen as seen in the histopathological observation on animals treated with ZNO NP as compared to that seen in animals administered only CCl<sub>4</sub> or only Doxorubicin indicated possibility of the ZnO NP being able to induce accelerated regeneration of the liver and spleen.

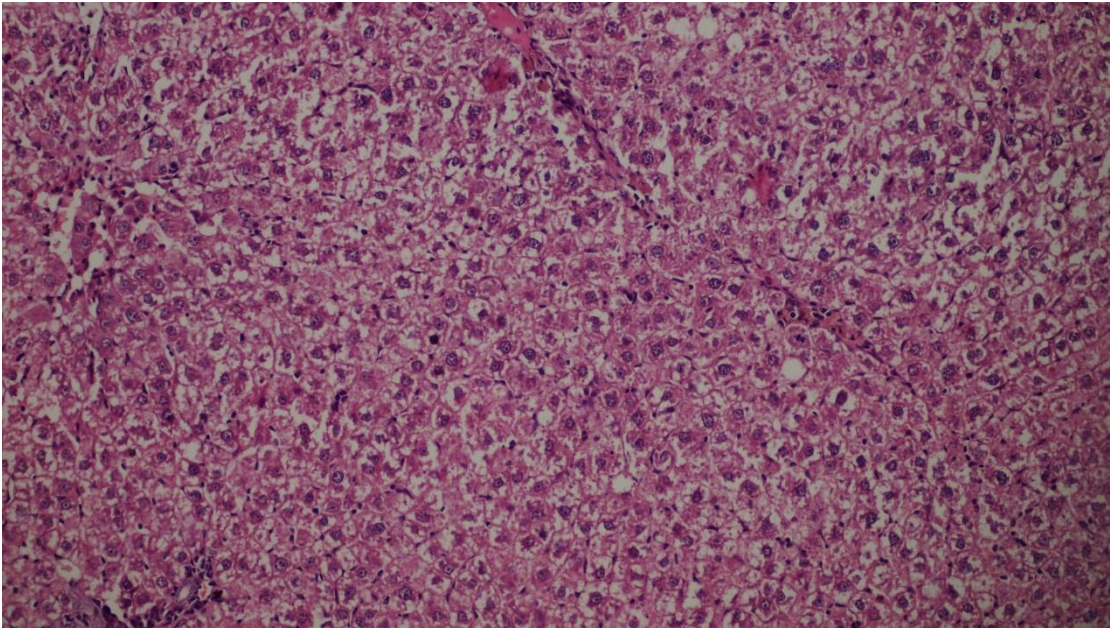


**Fig-1**(Group 5) : liver of rat showing severe vacuolar degeneration associated with proliferation of fibrous connective tissue surrounding the hepatic cells with dilated hepatic artery which is congested (H&E x400).

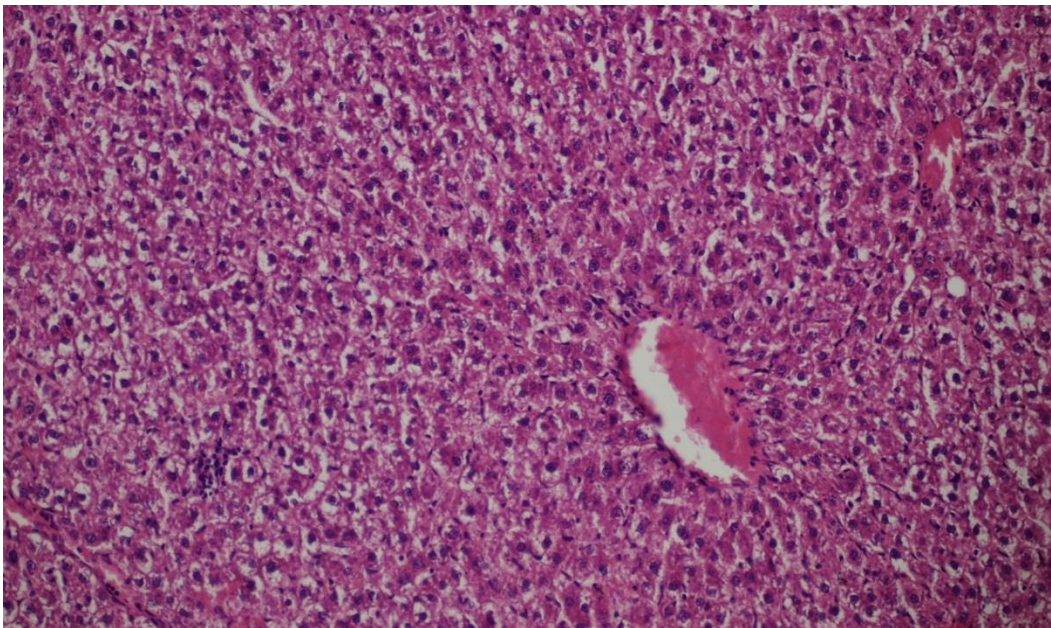


**Fig ( 2)**:Group 2: liver of rat showing vacuolar degeneration in some hepatic cells especially central vein ( H&Ex200).



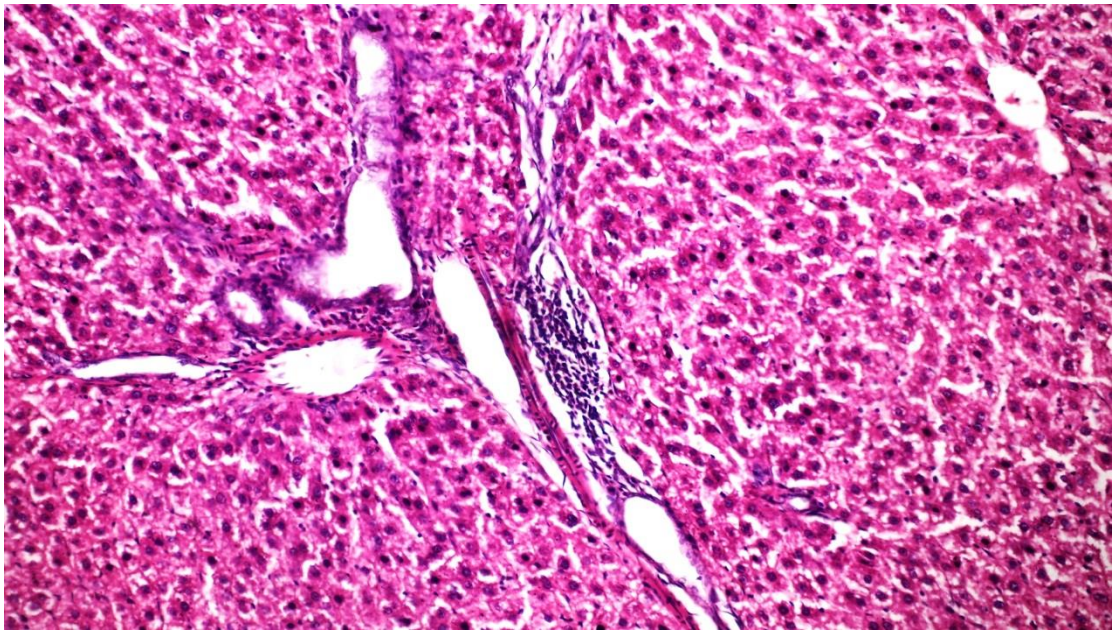


**Fig ( 3):** of Group 6 ( CCL4 group ): liver of rat showing severe vacuolar degeneration(H&E x 200).

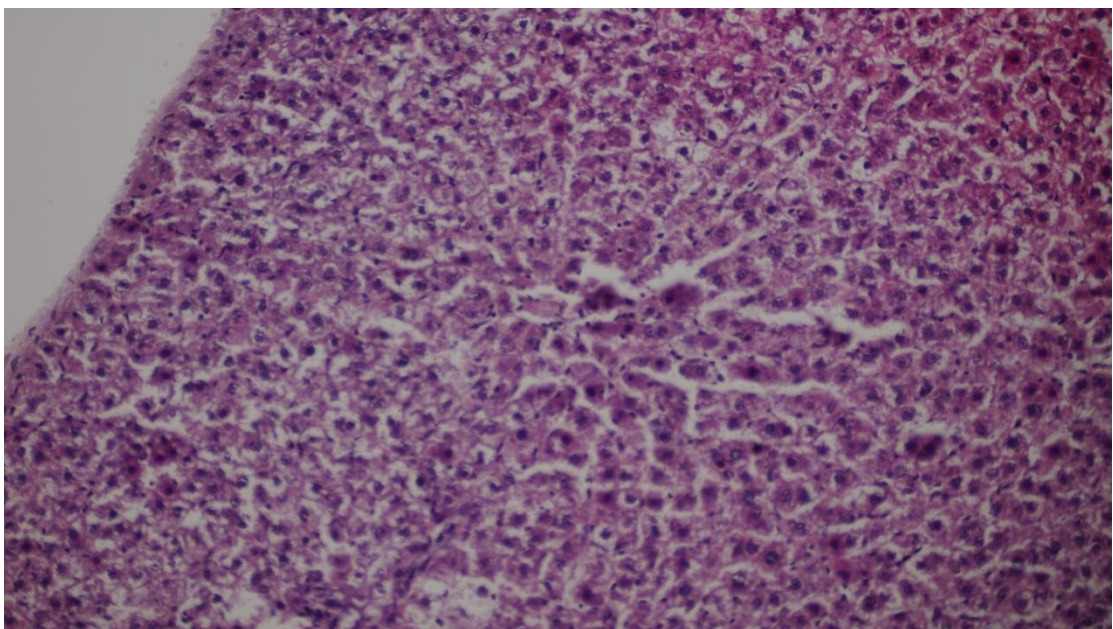


**Fig(4):** Group 7 ( CCL4 group ) liver of rat showing moderate vacuolar degeneration and congestion in some blood vessels ( H&E x 200).



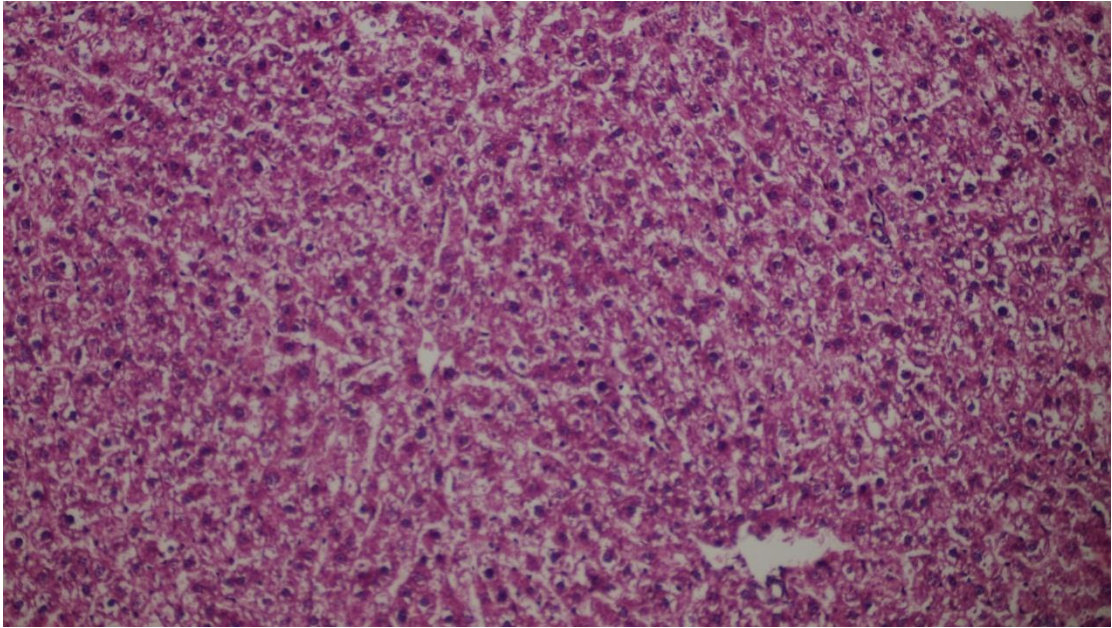


**Fig (5):** Group 7 ( CCL4 group ): liver of rat showing moderate proliferation of fibrous tissue and infiltration of mononuclear cells in portal area associated with mild dilatation of portal blood vessels (H&Ex200).

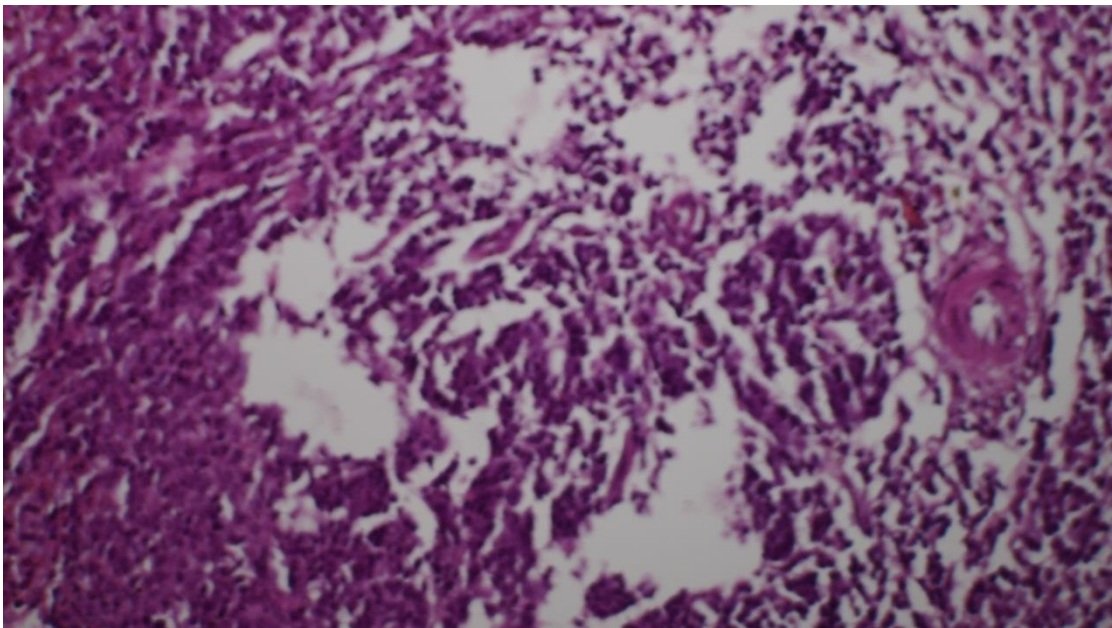


**Fig(6):**(Group 4)liver of rat showing mild vacuolar degeneration in some hepatic cells ( H&Ex 200).



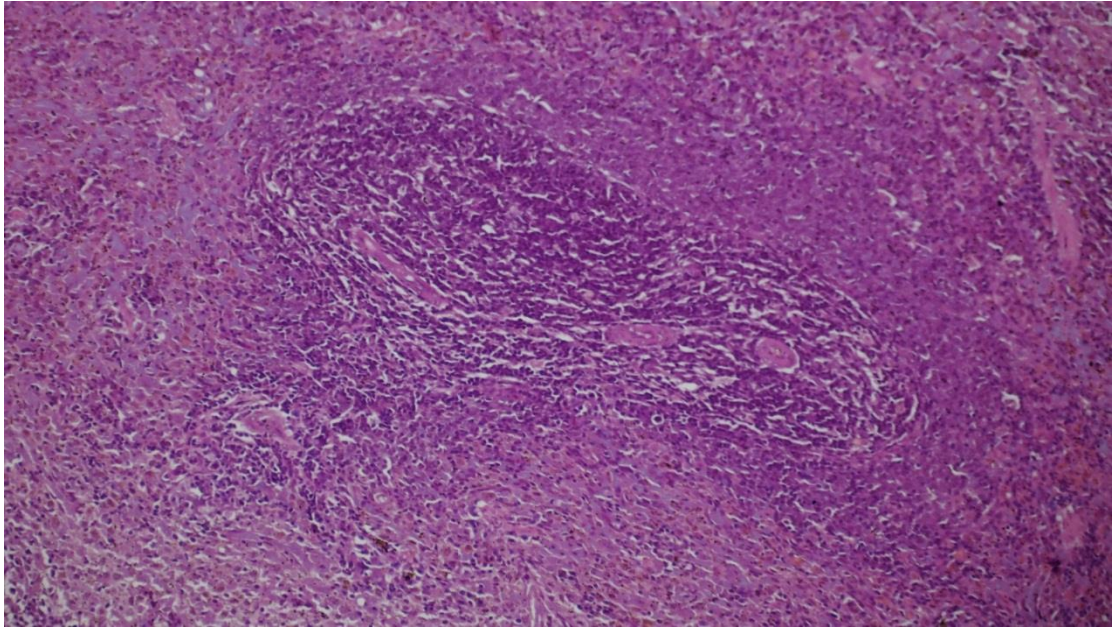


**Fig(7):** Group 8 ( CCL4 group ): liver showing moderate vacuolar degeneration of hepatic cells with some necrotic hepatic cells (H&Ex200).

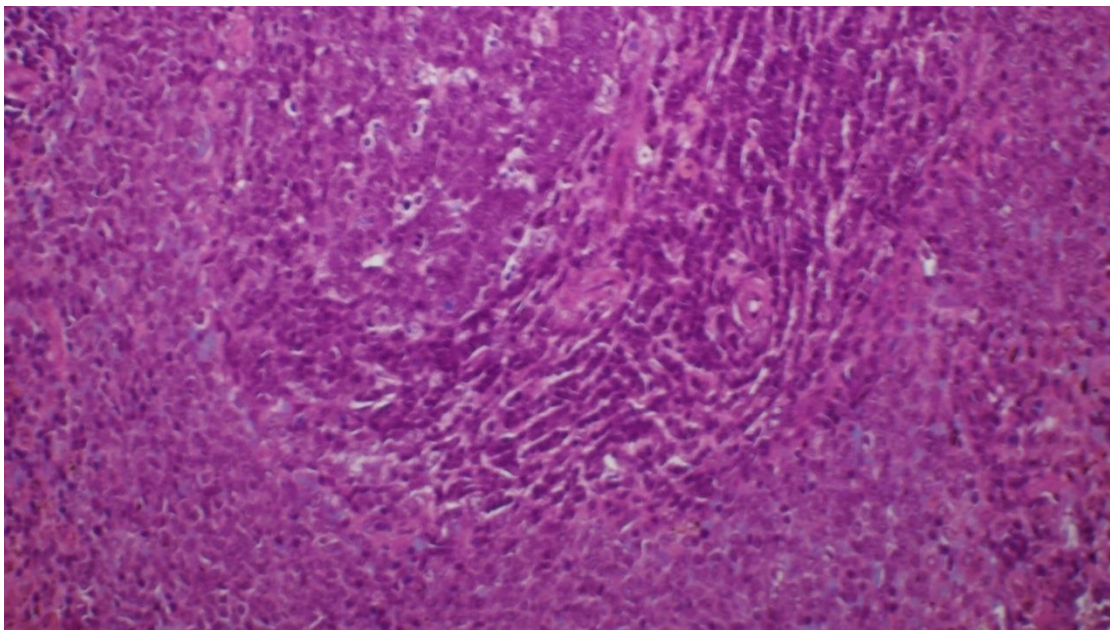


**Fig.(8):**Spleen of control positive group showing severe depletion in lymphoid elements in both white and red pulp especially in the center of lymphoid follicles ( H&Ex400).



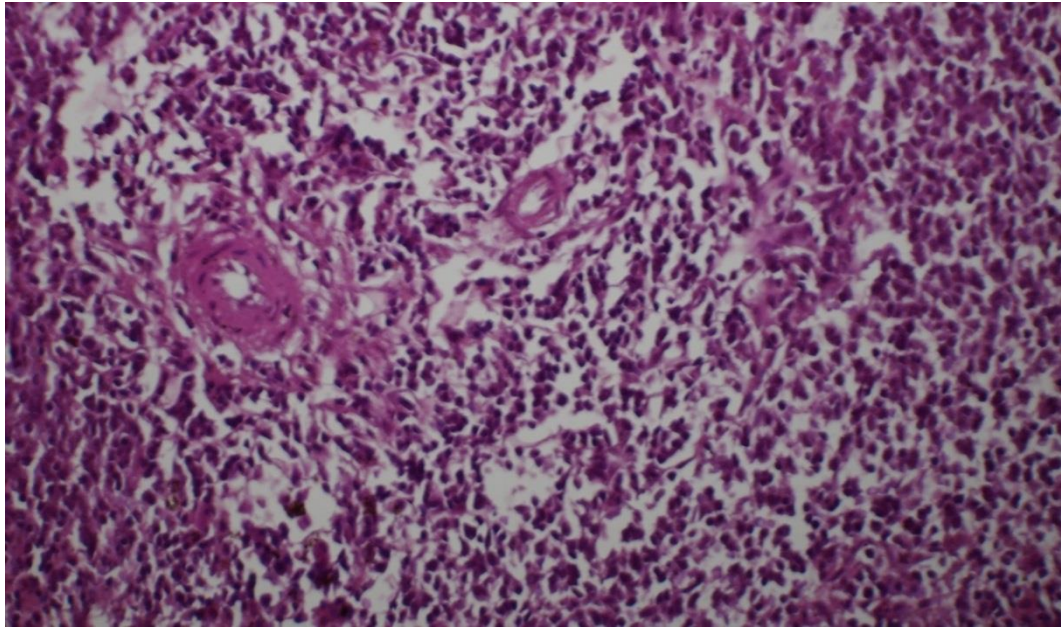


**Fig.(9):**Spleen from negative dox group revealing mild depletion in lymphocytes in both white and red pulb (H&Ex100).

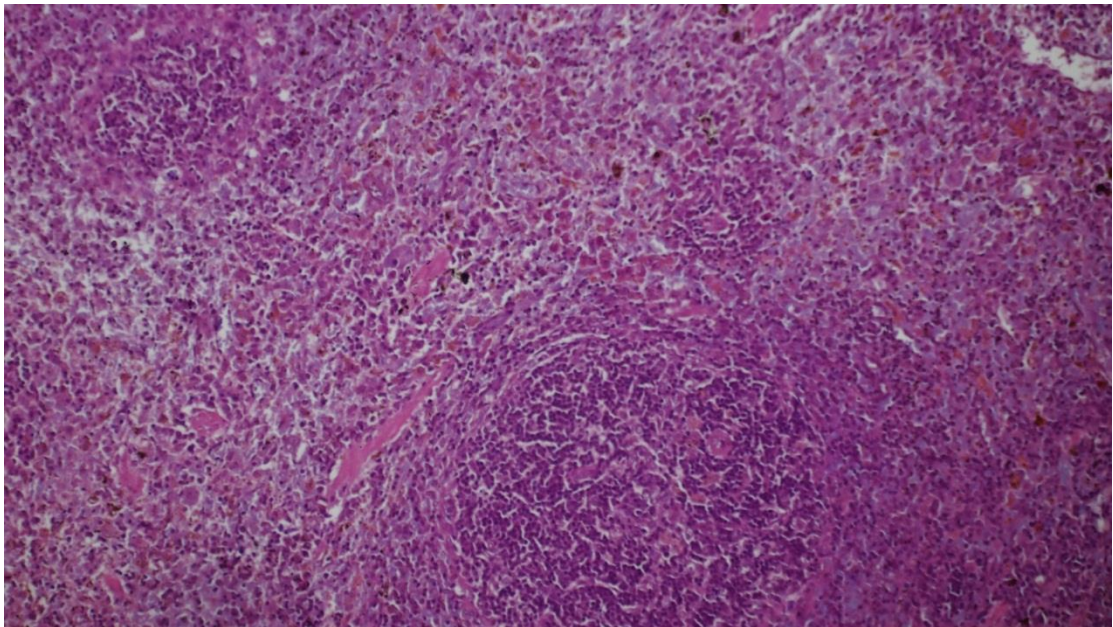


**Fig.( 10):**Spleen of positive dox.revealing hyperplasia of lymphoblast in the center of some white pulb (H&Ex400).



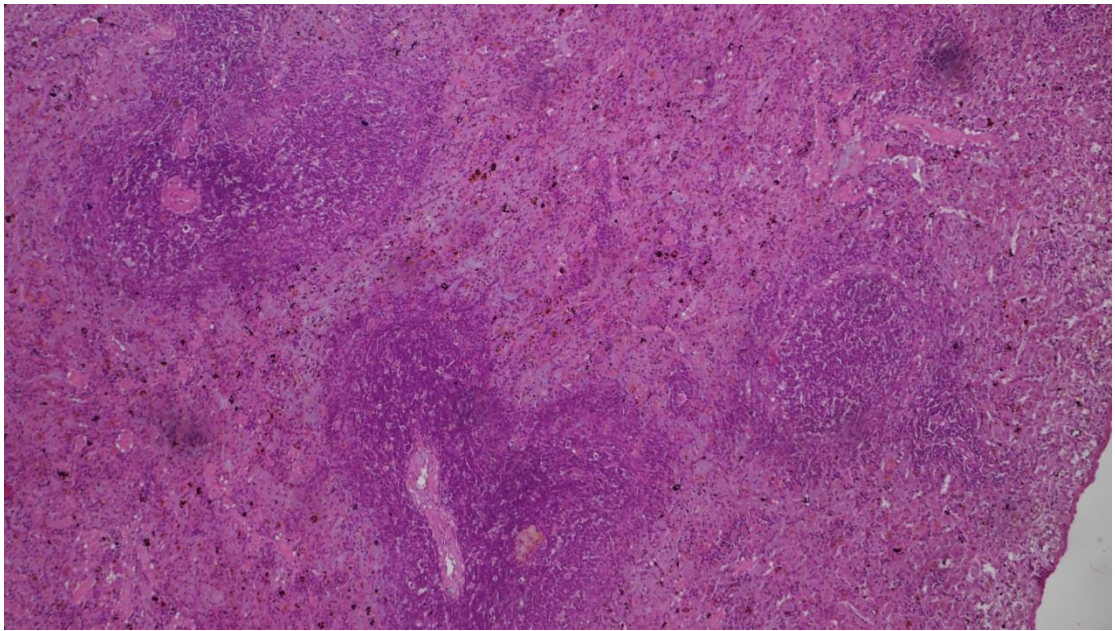


**Fig.(11):**Spleen of positive dox revealing moderate depletion in lymphocytes in white pulb( H&Ex400).

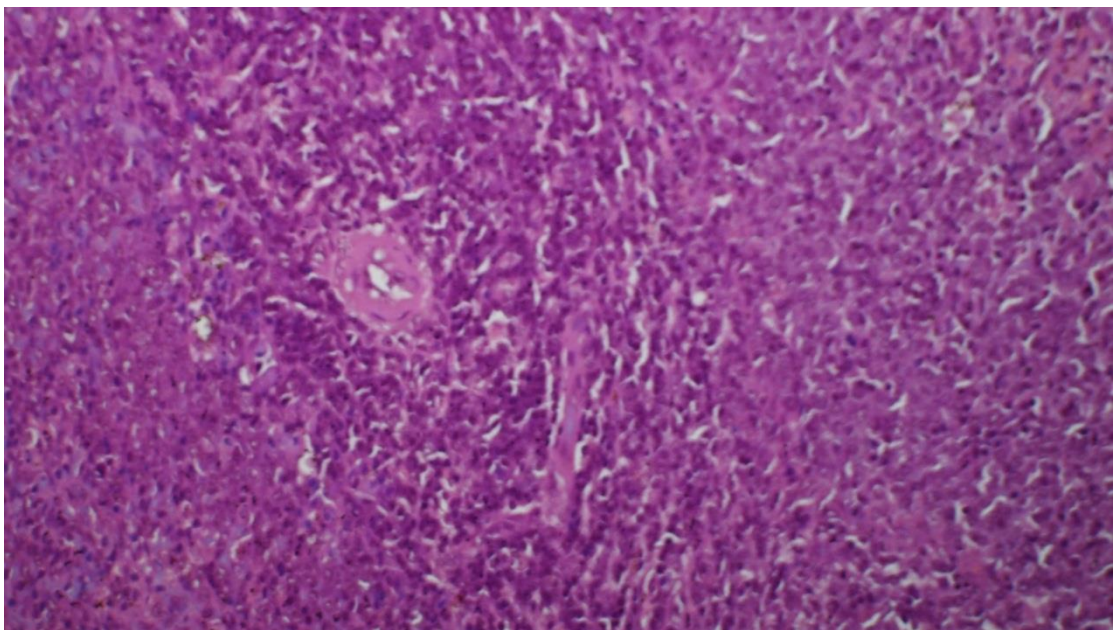


**Fig(12):**Spleen of negative zinc group revealing normal histological structure( H&Ex100).

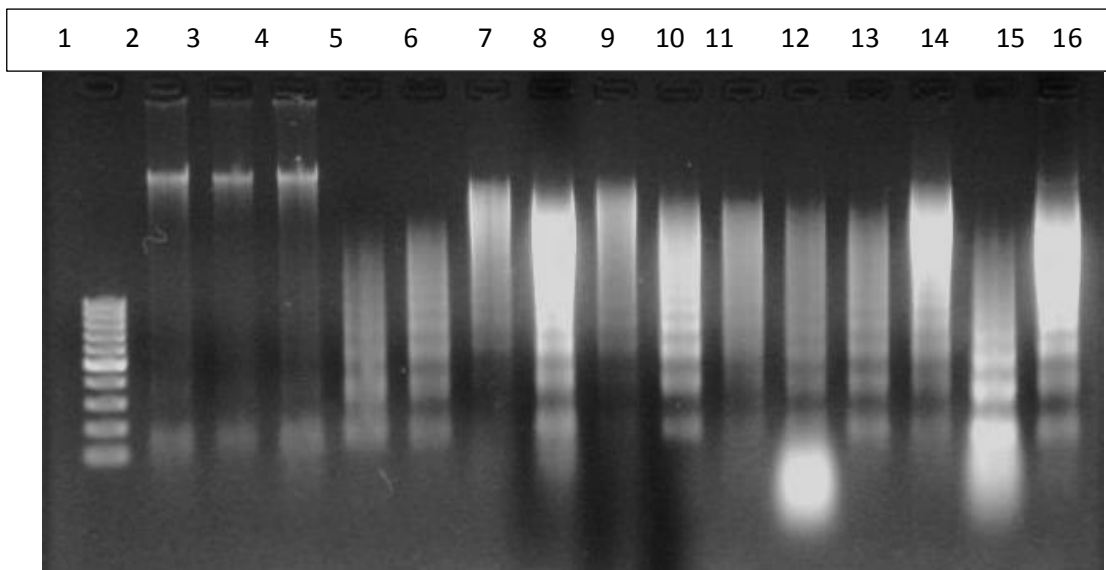




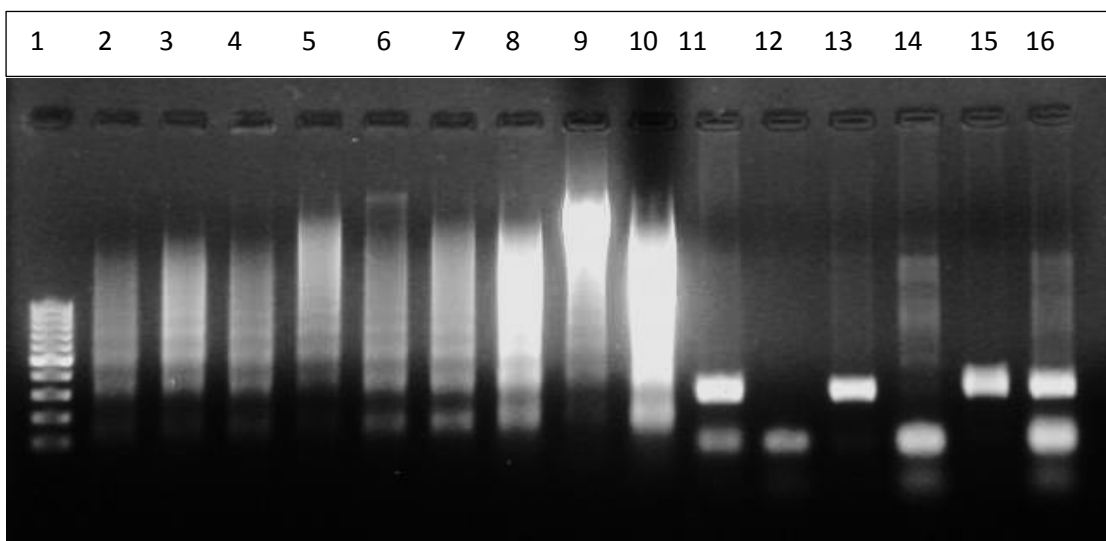
**Fig.( 13):** Spleen from positive zinc group revealing marked improvement in pathological changes ( H&Ex100).



**Fig.(14):**Spleen from positive dox and zinc group revealing marked improvement in pathological changes ( H&Ex400).



**Fig (15):** DNA fragmentation on agarose / ethyidium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. lane1:100-bp ladder. Lane (2-4) represents DNA isolated from control rats (G1) ; lane (5-11): received DOX (6 mg/kg/day) Group 2; Lane (12-16): received nZnO (5 mg/kg/day) Group 7 ( CCL4 group ).



**Figure (16):** DNA fragmentation on agarose / ethyidium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. Lane 1:100-bp ladder. Lane (2-7) represents DNA isolated from rats received nZnO (5 mg/kg/day) Group 3; lane (8-10): received DOX (6 mg/kg/day) Group 2; lane (11-16): received DOX (6 mg/kg/day) and nZnO (5 mg/kg/day) Group 4.

### Discussion

Nanotechnology allows the manipulation of materials at nanoscale level (1–100 nm), which enables precision engineering to control nanoparticles' (NPs') physicochemical properties, as well as their interactions with biological systems( **Lee et al., 2007; Wang, 2008**). Nanomedicine aims to overcome the problems related to human diseases at the nanoscale level, where most of the biological molecules exist and operate (**Wang and Thanou, 2010**).

This study was under taken to demonstrate the improvement ability of ZnO NP on liver damage induced by CCl<sub>4</sub> and the toxic effects of Doxorubicin in rats. The damage of the liver caused by CCl<sub>4</sub> and Doxorubicin was evident by DNA gel electrophoresis and histopathology. The results of ours study show that CCl<sub>4</sub> hepatotoxicity was effectively produced in rats received CCl<sub>4</sub>(**Fawcet and Bloom, 1994**). Similar observations have been earlier reported (**Gilani and Janbaz, 1995**).

Histological change like vacuolation of the hepatocytes and mild depletion in lymphocytes in both white and red pulb in spleen was observed in the group which received doxorubicin. This may be due to the hepatocytes are very active metabolic cells doxorubicin affects the cell cycle and kills the cells primarily by forming DNA adducts causing G2 arrest which leads to disturbances in its metabolic activities which in turn leads to shape distortion, possibly due to edema and accumulation of fluid within the cells. The disturbances in the function and shape of the hepatocytes due to the DNA damage in the liver cells provoked by doxorubicin ( **Hassan I El-Sayyad et al.2009** ). This finding is agreement with the work already reported (**Mohammed et al., 2013**).

Histological changes of the liver of rats showed moderate infiltration of mononuclear cells in portal area and accumulation of inflamatory cells around portal area with congestion in portal veins were observed in the group which received ZnO NP. This may be due to the toxicities effect of ZnO NP This effect linked to their physicochemical properties, such as dissolution into ionic Zn and their ability to generate ROS(**Xia, et al.,2011**). ZnO ENPs can directly generate ROS, because they present a significant number of electron–hole pairs (e–h+). The electrons and holes can react with the oxygen and hydroxyl ions, respectively, in the ZnO ENPs' surrounding aqueous environment. This produces highly reactive free radical compounds, including the superoxide anion (from electrons) and hydroxyl radicals (from holes)( **Pacchioni, 2003; Circu and Aw, 2008 ; Rasmussen et al., 2010**). The engulfed ZnO ENPs would have dissolved, releasing zinc ions, which may have caused toxicity to the surrounding hepatocytes. This “Trojan horse” scenario is consistent with a study investigating the effect of silica ENPs on Kupffer cell-mediated hepatic injury(**Chen et al., 2013** ). This is consistent with reported findings that NP-induced cytotoxicity requires NP contact with cells but is independent from the concentration of free zinc ions (**Moos et al., 2010** ) However, other studies report



that dissolution of ZnO NPs into free zinc ions is a major component of cytotoxicity (Xia et al., 2008; Sasidharan et al., 2011). This finding is in agreement with cytotoxicity of ZnO NPs against certain human cells reported in vitro (Lin et al., 2009; Akhtar et al., 2012). On the other hand, There is no effect in spleen in group which received ZnO NP only. And ZnO NP when used followed by Dox there are improvement in the histological change revealed by CCl<sub>4</sub> and Dox. DNA damage reduced when ZnO NP used followed by Dox. Use of ZnO NP showed marked repairing potential against CCl<sub>4</sub> and Dox. This improvement of histological change may be due to NPs are believed to be dependent on their physicochemical characteristics (Nel et al., 2006) . For example, ZnO NPs are cytotoxic, whereas their bulk counterparts are not (Reddy et al., 2007; Hanley et al., 2008) and their cytotoxicity is further improved with a decrease in NP size (Reddy et al., 2007; Nair et al., 2009; Hanley et al., 2009). ZnO NP size-dependent cytotoxicity (Nie et al., 2009; Nabeshi et al., 2010) but data on the effect of surface charge on ZnO NP preferential cytotoxicity remain controversial (Wilhelm et al., 2003; Verma and Stellacci, 2010; Baek et al., 2011). The mechanism of action of ZnO NPs is the generation of reactive oxygen species (ROS) (Xia et al., 2006). ROS are naturally produced during cell metabolism, and their concentration is tightly controlled by cellular antioxidant systems (eg, glutathione [GSH], superoxide dismutase, catalase). These components of the cell's antioxidant system are important for normal cell function, and physiological levels of ROS can act as second messengers and activators of cellular pathways (Hamanaka and Chandel, 2010). A better understanding of the underlying mechanisms of NP-induced ROS will allow for rational engineering of NPs that provide the greatest therapeutic benefit with minimum undesirable effects.

The repairing potential of ZnO NP against CCl<sub>4</sub> and Dox may be due to the tumor suppressor gene p53 is able to activate cell-cycle checkpoints, DNA repair, and apoptosis to maintain genomic stability (Sherr, 2004). In the presence of DNA damage, p53 protein triggers cell-cycle arrest to provide time for the damage to be repaired or for self-mediated apoptosis (Ahamed et al., 2008; Farnebo et al., 2010). The bax/bcl-2 protein ratio determines the life or death of cells in response to an apoptotic stimulus. The life or death of cells in response to an apoptotic stimulus. A higher level of bax/bcl-2 ratio decreases the resistance to apoptotic stimuli, leading to apoptosis (Chougule et al., 2010). The bcl-2 protein has an antiapoptotic effect, whereas the bax is known for proapoptotic activity. It has also been well documented that signaling pathway leading to apoptosis involves the sequential activation of cysteine proteases known as caspases (Tang et al., 2010; Ahamed et al., 2011). The expressions of both mRNA and protein levels of tumor suppressor gene p53 and proapoptotic genes (bax and caspase-3) were upregulated while the expression of antiapoptotic gene bcl-2 was downregulated in cancer cells treated with ZnO NPs

(**Mohd Javed Akhtar et al., 2012**). In the presence of DNA damage or cellular stress, p53 triggers cell-cycle arrest to provide time for the damage to be repaired or for self-mediated apoptosis(**Farnebo et al., 2010; Gopinath et al., 2010**) also showed that bax is up regulated by p53.

This results agree with **Rasmussen et al., (2010)** who reported that relevance for biomedical applications where improved approaches for drug targeting and drug delivery are needed. While several nanomaterial-based drugs such as Abraxane® and Doxil® are already on the market (**Nie et al., 2007; Rasmussen et al., 2010**) toxicity issues of NPs remain of concern (**Nel et al., 2006**). **Ostrovsky et al.,( 2009)** have reported that the ZnO NPs exerted cytotoxic effect on several human glioma cell lines (A172, U87, LN308, LN18, and LN229), and no cytotoxic effect was observed on normal human astrocytes. ZnO NPs exhibited a preferential ability to kill human myeloblastic leukemia cells (HL60) as compared with normal peripheral blood mononuclear cells(**Hanley et al., 2008; Premanathan et al., 2011**) also observed that ZnO NPs exhibit a strong preferential ability to kill cancerous T cells compared with normal cells. The cytotoxicity of ZnO NP can represent a desirable quality if it can be tailored to be specific against pathogenic cells. ZnO NPs exhibit cytotoxicity against cancerous cells at concentrations producing negligible effects on normal cells of the same lineage (**Hanley et al., 2008; Sasidharan et al., 2011; Akhtar et al., 2012**). Studies have shown that ZnO nanoparticles exhibit a high degree of cancer cell selectivity with the ability surpass the therapeutic indices of some commonly used chemotherapeutic agents in similar ex vivo studies (**Hanley et al., 2008; Wang et al., 2009**).

#### **Conclusion :**

Thus, it can be concluded that the reduce DNA damage and improved histology of the liver and spleen as seen in the histopathological observation on animals treated with ZNO NP as compared to that seen in animals administered only CCl4 or only Doxorubicin indicated possibility of the ZnO NP being able to induce accelerated regeneration of the liver and spleen.

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