Assiut Univ. J. of Zoology *Printed ISSN 1687-4935*
48(2), pp.36-59 (2019)

AMELIORATING EFFECTS OF THYMOQUINONE AND N-ACETYLCYSTEINE AGAINST URANIUM INDUCED HEPATOTOXICITY IN RATS

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Received: 8/5/2019 **Accepted: 13/6/2019 Available Online:1/12/2019**

Depleted uranium (DU) is a major health hazard environmental pollutant that is used primarily in military activity. Considerable evidence suggests the imuno-, geno- and reproductive toxicity of uranyl acetate (UA), a soluble salt of DU. A broad spectrum of data in literature indicates the antioxidant, antiapoptotic and cytoprotective properties of thymoquinone (TQ) and N-acetylcysteine (NAC). Therefore, we examined the ability of the two antioxidants, TQ or NAC to ameliorate UA hepatotoxicity in adult Wistar rats. The rats were injected intraperitoneally with a single dose of uranium as uranyl acetate (UA) (1 mg/kg body weight) alone or in combination with subsequent supplementation with NAC or TQ for 25 days. The obtained results showed that NAC or TQ administration in UA intoxicated rats ameliorated the adverse changes in the liver biochemical indices and hepatocellular features. They decreased alanine amino-transferase and aspartate amino-transferase; icreased glutathione and nitric oxide levels and the activities of superoxide dismutase and catalase, however, they decreased lipid peroxides and carbonyl protein content compared with UA group. In addition, the

present data indicated that TQ provided stronger protection against UA induced hepatotoxicity than NAC.

Key Words: Uranyl acetate - hepatotoxicity - thymoquinone - N-acetylcysteine and hepatocellular.

INTRODUCTION

The level of radiation increases daily due to rapid technological advancement; therefore there is an urgent need to protect human, animals, and even plants against its harmful effects **(Painuli and Kumar, 2016).** Radiation is categorized as either ionizing or nonionizing **(Groen *et al*., 2012).** It is well known that ionizing radiation is a radiation with enough energy so that during an interaction with an atom, it can remove tightly bound electrons from the orbit of an atom, causing the atom to become charged or ionized. There are three types of ionizing radiations: Alpha (α), Beta (β) and Gamma (γ) radiation **(Painuli and Kumar, 2016).** Uranium is an alpha-emitter and although unable to penetrate the outer skin layer, howver can cause irreversible cellular damage after internal contamination **(Vanhoudt *et al*., 2010).** Alpha particles react directly with DNA to generate double-strand breaks or interact with water to produce hydroxyl radicals, both of which negatively affect biological materials **(Jostein *et al*., 2010).**

Any disturbance in the redox state of an organism or a disturbance in the balance between production of reactive oxygen species (ROS) and endogenous antioxidant defense known as oxidative stress (OS) which leads to oxidation of lipids, proteins, and DNA in ways impairing cellular functions **(Burke and Fitzgerald, 2003).** Interestingly, ROS-induced damage can be alleviated using certain substances known as antioxidants **(Lalhminghlui and Jagetia, 2018)**. ROS are removed by antioxidants, such as GSH, vitamins C, A and E, and enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and thioredoxin **(Marí *et al*., 2010 and Serviddio *et al*., 2013).** In addition, lipid peroxidation (LPO) is one of the major consequences of OS **(Ozgur *et al.,* 2010)**, and MDA, an end product of lipid peroxidation, has been used as an index of oxidative damage **(Gunes *et***

***al*., 2008).** Furthermore, NO is believed to participate in the regulation of oxidation/reduction potential of various cells and may be involved in either the protection against or the induction of OS within various tissues, depending upon its concentration **(Bland, 1995).** Moreover, carbonylation not only had been used for decades as a biomarker of OS but also as a hallmark of many diseases, such as cancer **(Martin *et al*., 2014).** Carbonylation of biomolecules results from reactions with diverse forms of ROS **(Kuzmic *et al.,* 2016).**

Exposure to radiation changes the antioxidant levels in the body (**Nair *et al*., 2001**). For this reason, compounds with antioxidant properties have been shown to prevent the deleterious effects of ionizing radiations in living systems and bio-molecules due to their ability to scavenge free radicals **(Mathew *et al*., 2007).** TQ is the most abundant constituent of the volatile oil of *Nigella sativa* seeds (**Darakhshan *et al*., 2015).** Researchers demonstrated that TQ is hepatoprotective, anti-inflammatory, antioxidant, cytotoxic and anti-cancer chemical **(Khader and Eckl, 2014). Guida *et al.,* (2016)** provide evidence for the beneficial effects of TQ as an effective radioprotective candidate that enhances cellular immunity.

NAC, a thiol reducing agent, is a naturally occurring compound found in several vegetables, including garlic, onion (**Hsu *et al*., 2004),** peppers and asparagus **(Demirkol *et al*., 2004).** It has antioxidant, antiangiogenic, and anticarcinogenic properties **(Tosetti *et al*., 2002).** It is well established that NAC is a powerful antioxidant that is essential in liver detoxification **(Ozgur *et al*., 2010)**. However, there is no available literature highlighted the potential radioprotective effects of TQ or NAC on UA irradiated liver, therefore the aim of this study is to investigate this issue through investigating the changes in the liver function enzymes, oxidant/antioxidant balance and histoarchitecture in male Wistar rats.

Materials and Methods

I- Chemicals

Uranyl acetate dehydrate (UA), N-Acetylcysteine (NAC) and thymoquinone (TQ) were purchased from Sigma-Aldrich (St Louis, MO, USA). Kits used for estimation of ALT (Alanine amino-transferase), AST (Aspartate amino-transferase) and total antioxidant capacity were obtained from Company of Spectrum Diagnostics, Egypt. All other chemicals and reagents were of the highest purity commercially available.

II- Animals

Male rats of Wistar strain (n = 40) weighing (200 -250 g) were used in this study. Rats were purchased from the animal house of the Faculty of Medicine, Assuit University, Assuit, Egypt. The experimental procedures were conducted accordance with the animal care guidelines of the National Institutes of Health. Rats were housed in stainless steel cages at room temperature and maintained under light/dark cycles consisting of 12 h of light and 12 h of dark cycle. They were fed a standard commercial pellet diet and water throughout the experiment period. Animals were randomly divided into 4 groups of 10 animals each. **Group I:** Served as a control group. **Group II:** Received uranyl acetate (UA) dissolved in distilled water and injected intraperitoneally with a single dose (1 mg/kg body weight) according to a previous study **(Barber *et al.*, 2005)**. **Group III:** Received the same single dose of UA followed by subsequent daily intraperitoneal injection with NAC (100 mg/ kg/ body weight for 25 day) **(Prakash and Kumar 2009). Group IV:** Injected with the previously mentioned dose of UA followed by subsequent daily oral supplementation with TQ (20 mg/ kg/ body weight for 25 day) **(Badr *et al.*, 2013).**

III- Sample collection

The experiment was terminated at the end of 25 days. After an overnight fast, blood samples were taken from retro-orbital vein of each rat and immediately collected into EDTA tubes then centrifuged at 4000×*g* for 10 min at 4° C for preparation of plasma samples. For the histological

investigations, pieces of liver were fixed immediately in 10% neutral buffered formalin. For the subsequent biochemical assays, 0.5 g of each tissue was homogenized in 5 ml (0.1 M) phosphate buffer (pH 7.4) using homogenizer (IKA Yellow line DI 18 Disperser, Germany). The homogenates were centrifuged at 6,000 rpm for 1 hour at 4 °C and the supernatant cytosols were kept frozen at -20 °C until use.

IV- Measurement of liver enzymes

ALT and AST were determined in the plasma by enzymatic commercial kits according to the procedure of **Young (1990)** and expressed as U/L.

V- Antioxidant assays: Glutathione (GSH) was determined using the method of **Beutler *et al.* (1963).** Catalase (CAT) activity in tissue cytosol was determined based on its ability to decompose hydrogen peroxide **(Luck, 1963).** Superoxide dismutase activity in tissue cytosols was determined according to its ability to inhibit the autoxidation of epinephrine at alkaline medium according to the method of **Misra and Fridovich (1972)**. Total protein was measured as described by **Lowry *et al.* (1951).**

VI- Oxidative stress assays: Carbonyl protein (CP) and lipid peroxides (LPO) were measured according to the methods of **Stadtman and Levine (2000)** and **Ohkawa *et al.* (1979)**, respectively. Nitric oxide (NO) was assayed by Griess reagent according to the method of **Ding *et al.* (1988).**

VII- Histopathological examination

Specimens from liver tissues were fixed in 10 % buffered formalin, dehydrated in alcohol, cleared with xylene and embedded in paraffin wax. Four micron sections were cut and stained with haematoxyline and eosin (HE) and examined by light microscopy.

VIII- Statistical analysis

The results had been analyzed by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test as a post-test. These analyses had been carried out using GraphPad Prism program for windows, version 5.0 (San Diago CA. USA). The significance difference between groups had been accepted at $p<0.05$, 0.01 or 0.001, and the data were expressed as mean ± Standard error (SE).

RESULTS

1) **Plasma ALT and AST activity**

Data obtained from **(Fig. 1)** indicated that the activity of ALT and AST (U/L) in the plasma of rats treated with UA alone significantly ($P < 0.001$ and $P < 0.05$, respectively) increased in comparison with the control group. However, the activity of ALT and AST was significantly decreased in UA+NAC and UA+TQ group ($P < 0.001$and $P < 0.05$, respectively).

2) **Catalase activity (CAT)**

Table 1 showed a significant ($P < 0.01$) decrease in the liver CAT activity of rats intoxicated with UA compared with the control rats. Treatment of UA intoxicated rats with NAC or TQ significantly ($P < 0.05$) increased CAT activity in comparison with the untreated rats.

3) **Glutathione (GSH)**

The liver content of GSH as nmol/mg protein **(Table 1)** was significantly ($P < 0.01$) decreased in UA group in comparison with the control group. On the other hand, treatment of UA challenged rats with NAC numerically increased the concentration of GSH but did not reach the significant level. Nevertheless, treatment of UA challenged rats with TQ resulted in a significant ($P < 0.05$) increase in GSH concentration compared to the UA group.

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4) **Superoxide dismutase activity (SOD)**

The data obtained from **(Table 1)** indicated that the liver SOD activity (U/min/mg protein) decreased significantly (P < 0.05) in UA group compared with the control group. Treatment of UA intoxicated rats with NAC significantly (P < 0.05) increased the activity of SOD. Interestingly, the administration of TQ in combination with UA also played a significant (P < 0.05) role in reducing the decrease in SOD activity in comparison with the UA group.

5) **Lipid peroxidation (LPO)**

As compared to the control group, the concentration of LPO (nmol MDA/mg protein) significantly (P < 0.01) increased in the liver of rats exposed to UA. However, LPO concentration decreased numerically in the liver of UA exposed rats treated with NAC but did not reach the significant level. Nevertheless, supplementation of TQ in combination with UA caused a significant (P < 0.05) decrease in LPO concentration **(Table 2)**.

6) **Carbonyl protein (CP)**

Table 2 showed a significant (P < 0.001) increase in CP content in the liver of UA challenged rats compared to the control group. However, treatment of UA challenged rats with NAC numerically decreased the content of CP in liver without reaching the significant level. Nevertheless, treatment of UA challenged rats with TQ resulted in a significant (P < 0.001) decrease in the content of CP.

7) **Nitric Oxide (NO)**

Data obtained from **Table 2** indicated that UA administration significantly (P < 0.05) decreased NO content (nmol sodium nitrite /mg protein) in the liver. Treatment of UA intoxicated rats with NAC caused a non significant increase in NO content in comparison with UA group. On the other hand, supplementation of UA intoxicated rats with TQ significantly (P < 0.05) restored

the level of NO in the liver nearly similar to the normal value observed in the control rats.

8) **Histopathological examination**

Examination of the liver tissues of the control rats revealed a normal histoarchitecture **(Fig. 2A)**. However, intoxication of rats with UA resulted in vascular and cellular changes. The vascular changes appeared in the form of congestion in the central veins and blood sinusoids **(Fig. 2B)**. The hepatocytes showed degenerative changes extended to necrosis and infiltration by mononuclear inflammatory cells **(Fig. 2C, D)**. TQ treated group showed normal histological appearance **(Fig. 2E)**, while NAC treated group showed congestion and Kupffer cell proliferation **(Fig. 2F).**

Discussion

The increase in the plasma levels of AST and ALT in UA challenged rats is corresponding with that previously reported **(Yuan *et al.*, 2017)**. The increase in theses pathogenic markers indicated cellular leakage and loss of functional integrity of the liver cell membrane secondary to free radicals-mediated lipid peroxidation **(Yapar *et al.*, 2010; Al-Malki and Sayed, 2014)**, and matched well with the degenerative changes observed in the liver sections in the present study. The hepatoprotective effects of TQ appeared obviously as evident by a significant decrease in the plasma levels of AST and ALT compared with the intoxicated group. This finding is in harmony with the ability of TQ to ameliorate lead-induced hepatotoxicity through the suppression of lipid peroxidation and elevation of total antioxidant status in the liver **(Mabrouk et *al.*, 2016)**. TQ inhibits the covalent binding of free radical products to intracellular macromolecules such as lipids **(Mansour, 2000)**. TQ protected isolated rat hepatocytes against tert-butyl hydroperoxides-induced hepatotoxicity by preventing the depletion in intracellular glutathione and thus maintaining the stability of cell membrane **(Daba and Abdel-Rahman, 1998)**.

In this study, the marked decrease in the CAT activity in the liver of UA challenged rats compared with the control group is contradictory with the results of **El-Missiry *et al.* (2007)** who found a significant increase in the CAT activity in liver of irradiated rats following 5 days of exposure. This controversy may attribute to differences in the dose and duration of radiation. UA obviously reduced the activity of CAT, possibly due to oxidation of sulfhydryl groups of the enzyme active sites, or due to other structural and functional changes induced in the enzyme by ionizing radiation **(Zigman *et al.,* 1996; Wu *et al.,* 2008).**

The results of the present study indicated that TQ induced a significant increase in the liver CAT activity compared with UA group similar to **Mansour (2000),** and can be explained by up-regulation of CAT gene expression **(Ismail *et al.*, 2016)**.

The significant decrease in the liver GSH content in UA group compared with the control group is consistent with that found in isolated hepatocytes and brain mitochondria (**Pourahmad *et al.*, 2006; Pourahmad *et al.*, 2011; Shaki *et al.*, 2013**). UA increased ROS formation and GSH reacts with ROS and is converted to GSSG that finally resulted glutathione depletion in the hepatocytes **(Pourahmad *et al.*, 2011)**.

Many studies have revealed the importance of SOD in protection of normal tissue from the harmful effects of ionizing radiation **(Holley *et al.*, 2014)**. In the present study, liver SOD activity decreased in UA group compared with control group. This result is in agreement with that of **Ozgur *et al.* (2010)** who found a significant decrease in the activity of SOD in the liver of guinea pigs after radiofrequency radiation exposure. On the other hand, uranium administration led to a significant enhancement in SOD activity in both kidney and testis of rats **(Linares *et al.,* 2006).** The variation in the dose rather than duration of exposure may be the determining factor underlined this conflict in the pattern of SOD response.

The present results showed that treatment with natural antioxidants NAC or TQ increased the activity of liver SOD compared with UA group. Similarly with our result, NAC treatment prior to radiation was found to significantly increase the antioxidant status when compared with irradiated group **(Mansour *et al.*, 2008).** The superoxide radical scavenging property and protective role of TQ in saving SOD from glycation by reducing structural changes seemed to the causative agents underlying the maintenance of SOD activity in spite of UA pro-oxidative activity **(Nagi and Mansour, 2000; Anwar *et al.*, 2014)**.

Data obtained from the present study indicated that liver LPO content increased in UA group compared with the control group in parallel with that observed in diazinon-induced hepatotoxic mouse model **(Nili-Ahmadabadi *et al.*, 2018)**. UA toxicity leads to hepatic overproduction of ROS which attack polyunsaturated fatty acids resulting in formation of LPO which alters the physical properties of cellular membranes and causes covalent modification of proteins and nucleic acids **(Pourahmad *et al.*, 2006; Gaschler and Stockwell, 2017)**.

Lipid radicals are believed to be formed by the reaction of hydroxyl radicals generated by ionizing radiation with polyunsaturated fatty acids **(Pandey and Mishra, 2000).** Thus, scavenging free radicals and inhibiting lipid peroxidation are likely key target activities for developing successful radioprotection strategies **(Hosseinimehr *et al.*, 2001).** In the present study, measurement of LPO provides evidence that both TQ had protective effects against UA intoxication in the liver tissues of rats. These findings are in harmony with **Mansour (2000)** who concluded that TQ is efficient cytoprotective agents against carbon tetrachloride-induced hepatotoxicity possibly through inhibition of the production of oxygen free radicals that cause lipid peroxidation. TQ activates SOD and glutathione transferase, increases GSH levels, and suppresses lipid peroxidation **(Schneider-Stock *et al.*, 2014).**

The results of this study showed that liver CP levels significantly increased in UA group compared with the control group in harmony with the results of **Lestaevel *et al.* (2015)** in the hippocampus of rats exposed to uranium. **Almroth *et al.* (2005)** found that the formation of carbonyl derivatives causes conformational changes, decreases in the enzymatic catalytic activity and ultimately results in breakdown of proteins by proteases**.** So, the significant inhibition of enzymatic antioxidants measured in this study in the liver of UA intoxicated rats (e.g., CAT and SOD) may be associated with the effects of UA on CP level.

The current study showed that treatment of UA challenged rats with TQ resulted in a significant decrease in the liver content of CP. This result may attribute to the strong antioxidant potential of TQ and its potent activity in scavenging free radicals as claimed by **Hosseinzadeh *et al.* (2007).**

The significant reduction in NO levels in the liver of UA group in comparison with the control group in the present experimental model is matched with that found following direct ingestion of contaminated soil by depleted uranium **(Hao *et al.,* 2012**). This is may be owing to inhibition of NO synthase pathway leading to decrease in endothelial NO synthase **(Dublineau *et al.,* 2006).** The pronounced decrease in the liver NO level, suggests dysfunction in vascular endothelium probably due to its inflammation supporting the vascular changes in the liver in the present study including congestion of central veins and blood sinusoids.

TQ supplementation to UA exposed rats caused an obvious increase in the liver NO level compared to the untreated rats. Although presence of several evidences indicating that TQ suppresses the expression of inducible nitric oxide synthase **(El-Mahmoudy *et al.*, 2002; Abdel-Zaher *et al.*, 2013**), the antioxidant capacity of TQ could decrease superoxide generation and consequently its interaction with NO to form peroxynitrite resulting in accumulation of NO.

Histopathological examination of HE-stained liver sections in our study revealed vascular and degenerative changes in harmony with what happen previously in U-intoxicated rats **(Yuan *et al.*, 2017).** The depletion in mitochondrial ATP which associated with intracellular acidosis and osmotic injury leading to plasma membrane lysis **(Pourahmad and O'Brien, 2000)**, and lysosomal membrane damage secondary to ROS attack **(Pourahmad *et al.*, 2006)** may contribute to the necrosis in hepatocytes.

The present study found that co-administration of TQ with UA returned the liver to the normal histological appearance, while NAC treated group still showed some histopathological changes in the form of congestion and Kupffer cell proliferation**.** The improvement in the liver histology of UA intoxicated rats following TQ treatment is in accordance with that observed in methotrexate-induced hepatotoxic rat model, and is most probably happen due to antioxidant, antinitrosative, anti-inflammatory, and antiapoptotic mechanisms (**El-Sheikh *et al.*, 2015**). The partial amelioration of UA hepatotoxicity by NAC supplementation is in consistent with that found in the hepatic ischemia-reperfusion rat model (**Smyrniotis *et al.*, 2005**). The inhibition of apoptosis and maintanenance of GSH homeostasis are suggested to play a key role in the hepato-protective ability of NAC (**Santra *et al.*, 2007**).

In conclusion, the present study showed that NAC or TQ supplementation effectively alleviated liver damage caused by UA. They had beneficial antioxidant effects against redox status and protected against hepatocellular damage caused by UA. Nevertheless, TQ supplementation was more effective than NAC in attenuation of UA-induced hepatotoxicity.

Acknowledgement:

The authors are gratefully indebted to Prof. Dr. Abdel-Hadi El-Kamel, Professor of Radiation Physics, Faculty of Science, Assiut University, for his constructive guidance and continuous help through this work.

Conflict of interest:

The authors declare that there are no conflicts of interests.

Author's contribution:

Waly H, Khaled E., El-Gamal H., designed the experiment, performed the practical work and wrote the paper, Hassanein KM., analyzed the H&E stained slides.

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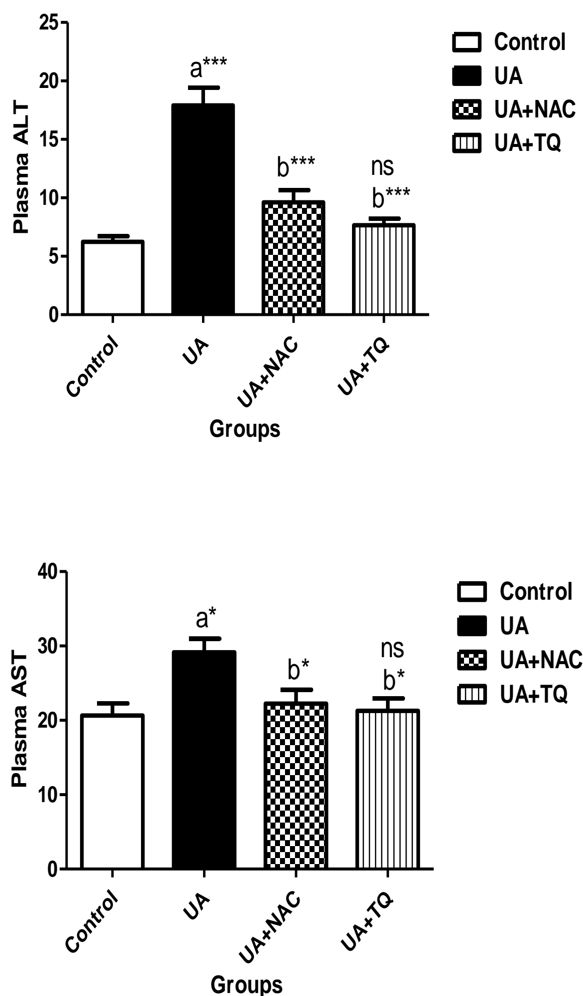

Fig. (1): Effect of treatment with UA, UA+NAC and UA+TQ on the activity of ALT and AST in the plasma of different experimental groups
Data are represented as Mean ± SE, n = 10.

Values represent means ± SE. a: significant difference between control and UA group. b: significant difference between UA and different treatments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns= non significance difference between (UA+NAC) and (UA+TQ) treated groups.

UA: uranyl acetate, NAC: N-acetylcysteine; TQ: thymoquinone; LPO: lipid peroxides, CP: carbonyl protein; NO: nitric oxide

Table (1): Antioxidant assay markers in the liver of different experimental groups

Group	CAT (U/mg protein)	GSH (nmol/mg protein)	SOD (U/min/mg protein)
Control	10.07± 0.5871	0.848±0.111	7.956±0.573
UA	7.975±0.2921^{a**}	0.456± 0.025^{a**}	5.214±0.423^{a*}
UA+NAC	9.727± 0.344^{b*}	0.6266±0.043	7.675±0.684^{b*}
UA+TQ	9.411± 0.299^{b*}	0.7317±0.058^{b*}	7.190±0.630^{b*}

Values represent means ± SE. n = 10

a: significant difference between control and UA group. b: significant difference between UA and different treatments. * P < 0.05, ** P < 0.01, *** P < 0.001

UA: uranyl acetate, NAC: N-acetylcysteine; TQ: thymoquinone; LPO: lipid peroxides, CP: carbonyl protein; NO: nitric oxide

Table (2): Oxidative stress assay markers in the liver of different experimental groups

Group	LPO (nmol/mg protein)	CP (nmol/mg protein)	NO (nmol/mg protein)
Control	0.160±0.007	12.92 ± 1.816	0.731±0.091
UA	0.274± 0.019^{a**}	56.43 ± 4.148^{a***}	0.467±0.042^{a*}
UA+NAC	0.2454±0.030	48.62 ± 3.428	0.371 ± 0.033
UA+TQ	0.191± 0.025^{b*}	29.91 ± 5.973^{b***}	0.667±0.048^{b*}

Values represent means ± SE. n= 10

a: significant difference between control and UA group. b: significant difference between UA and different treatments. * P < 0.05, ** P < 0.01, *** P < 0.001

UA: uranyl acetate, NAC: N-acetylcysteine; TQ: thymoquinone; LPO: lipid peroxides, CP: carbonyl protein; NO: nitric oxide

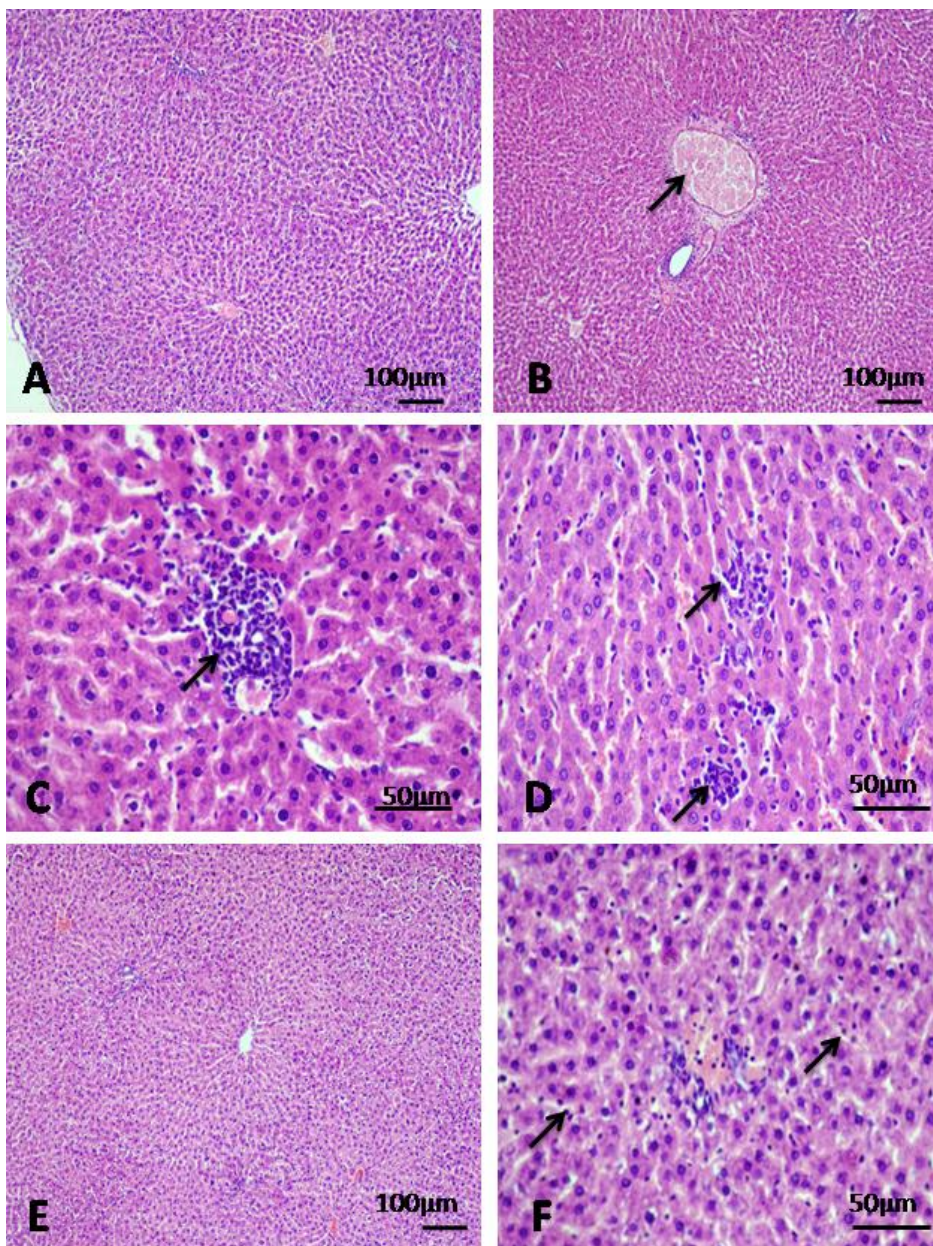

Fig. (2): A) Liver of control rats showing normal histological architecture. **B)** Liver of UA group showing congestion of the central vein (arrow), **C, D)** Degeneration and necrosis of hepatocytes with mononuclear cell infiltration (arrows). **E)** Liver of TQ group showing normal appearance. **F)** Liver of NAC group showing Kupffer cell proliferation (arrow). **HE**