Journal of Bioscience and Applied Research

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Folic acid ameliorates L-thyroxin induced hepatotoxicity and oxidative stress in albino rats

Somya Y. Shalaby[1], Saber A. Sakr[1], Ehab Tousson[2], Mohamed Rabea[1]

1. Department of Zoology, Faculty of Science, Menoufia University, Egypt
2. Department of Zoology, Faculty of Science, Tanta University, Egypt

(Corresponding author e-mail. sabsak@yahoo.com)

Abstract

Thyroid hormones have been known to regulate the energy metabolism of most tissues including liver. Alterations in their normal levels cause some biochemical and clinical abnormalities such as hypothyroidism and hyperthyroidism. The present study evaluated the effect of thyroid hormone, L-thyroxin on liver of albino rats. Additionally the ameliorating role of folic acid supplementation was investigated. Fifty male albino rats were randomly divided into five groups (group I, control; group II, folic acid; group III, L-thyroxin sodium administration (100 µg/kg / body weight); group IV, L-thyroxin and folic acid group and V, recovery group). The results showed that there were a significant increase in ALT, AST, MDA and nitric oxide in L-thyroxin treated rats as compared to control group. On the other hand, a significant decrease in glutathione (GSH) in L-thyroxin treated rats as compared to control group. Histological results showed that liver sections of L-thyroxin group showed histopathological lesions such as leucocytic infiltrations, congestion of central and portal veins and cytoplasmic vacuolation of hepatocytes with the presence of pyknotic nuclei, in addition to fatty infiltration. Immunohistochemical results revealed that strong positive expression of PCNA, P53, and Bcl-2 were detected in the liver section in L-thyroxin treated rats and recovered rats as compared to control and folic acid groups. However; mild to moderate positive expressions of PCNA, P53, and Bcl-2 were observed in rats treated with L-thyroxin and folic acid in liver section. This reflects oxidative stress associated with hyperthyroid state.

Keywords:Hyperthyroidism, liver, ALT, AST, oxidative stress, PCNA, P53 and Bcl-2.

1 Introduction

Thyroid hormones have been known to regulate basal metabolic rate for more than a century and are now recognized to control a bulk of physiological processes, such as growth, development, and metabolic rate (Brent, 2012). Thyroid hormones, previously thought not to affect spermatogenesis and male fertility, are now being recognized as having important role in spermatogenesis (Krajewska-Kulak and Sengupta, 2013). Alterations in their normal levels cause some biochemical and clinical abnormalities such as hypothyroidism and hyperthyroidism. Extended exposure to the treatment with L-thyroxin may alter thyroid activity by interfering with thyroid hormones synthesis, which provokes the disruption of thyroid axis, resulting in numerous abnormalities (Araujo et al., 2006). Hypothyroidism and hyperthyroidism are a result of an imbalance of thyroid hormone. Hypothyroidism is simply not enough thyroid hormone and hyperthyroidism is too much. Either imbalance affects the metabolism in the body. Thyroid hormones have a significant impact in the regulation of hepatic mitochondrial metabolism (Paradies et al., 1991). The liver is the major site for cholesterol and triglyceride metabolism and the thyroid hormones play an integral part in hepatic lipid homeostasis. Thyroid hormones increase the expression of LDL (low density lipoprotein) receptors on the hepatocytes (Ness et al., 1998).Also thyroid hormone (TH) increases the activity of lipid-lowering liver enzymes, resulting in a reduction in low - density lipoprotein levels (Ness and Lopez, 1995). Thyroid hormones also increase the expression of Apo-lipoprotein A1, a major component of high-density lipoprotein (Taylor et al., 1997). Clearly, the above effects

of the thyroid hormones could be beneficial in reducing the onset of atherosclerosis if they were elicited without the deleterious effects, particularly cardiac effects (Dillmann., 1990).

Folic acid is a water-soluble vitamin, which is essential in life. Numerous clinical trials using folic acid for prevention of cardiovascular disease, stroke, cognitive decline, and neural tube defects have been completed or are underway (Massoud et al., 2012). Folic acid and folate represent the synthetic form (folic acid) and the naturally occurring form (folate) of vitamin B9. Supplementation with folic acid has also been shown to reduce the risk of congenital heart defects, cleft lips, limb defects, and urinary tract anomalies (Goh and Koren, 2008).

Bazzano, (2011) reported that folic acid and vitamin B12 supplementation consumed before and during pregnancy may reduce the risk of heart defects in infants, taking folic acid does not reduce cardiovascular disease even though it reduces homocysteine levels. Also, it may reduce the risk for children to develop metabolic syndrome (Stewart et al., 2009). García-Miss et al. (2010) showed that folate deficiency may increase the risk of schizophrenia because, by increasing homocysteine levels, folate also increases interleukin 6 and tumor necrosis factor alpha levels, and these two cytokines are involved in the development of schizophrenia. The present study was designed to declare the effect of L-thyroxin on the liver functions, oxidative stress parameters, immunohistochemical observations. Additionally, the ameliorating role of folic acid supplementation was investigated.

2 Materials and Methods

Animals and treatment

Male albino rats (*Rattus norvigicus*) weighing (130 g ±10g) were used. They were obtained from Helwan laboratory farms, Egyptian Organization for Vaccine and Biologic Preparations. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard rodent diet (20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminzed starch; Egyptian Company of Oils and Soap Kafr-Elzayat Egypt) and water was available ad libitum. The temperature in the animal room was maintained at 23±2OC with a relative humidity of 55±5%. Light was on a 12:12 hr light -dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research approved by Menoufia University. The rats were randomly and equally divided into five groups (10 rats each).

Group 1 (G1): Rats of this group considered as control.

Group 2 (G2): Animals received folic acid (El Nasr Pharmaceutical Chemicals Co.) at a dose level of 8 mg/kg of body weight/ day) for three weeks from 3rd week to 6th week **(**Tousson et al., 2012).

Group 3 (G3): Rats of this group received of L-thyroxin sodium at a dose of 100 µg/kg / body weight daily for three weeks to induce hyperthyroidism (Sahoo et al., 2008).

Group 4 (G4): Rats of this group received L-thyroxin sodium (100 µg/kg/ body weight/day) for three weeks, followed by folic acid (8 mg/kg / body weight/day) for another three weeks.

Group 5 (G5): Rats of this group were received L-thyroxin sodium (100 µg/kg / body weight/day) for three weeks and left without treatment for another three weeks.

Biochemical studies

For biochemical measures, blood was collected from rats of different groups. Sera were acquired by centrifugation of the blood test and put away at - 20°C. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were resolved colourimetrically as indicated by Reitman and Frankel (1957). liver was quickly removed, weighed and stored at -20°C then 10%W/V homogenate was prepared by grand 0.3 g of tissue in 3ml of saline. Malondialdehyde (MDA) was estimated by the method of (Placeret al.1966), nitric oxide (NO) was measured by the method of (Miranda et al.2001) and glutathione (GSH) was estimated by the method of (Ellman,1959).

Histological study

Liver was immediately removed from dissected rats and were immediately fixed by immersion in 10% buffered formalin solution and left for 24-48 hours. The specimens were then dehydrated, cleared and embedded in paraffin. Sections of 5 µm thick were cut by mean of rotary microtome and stained with haematoxylin and eosin and examined under light microscope.

Immunohistochemical study

The immunostaining was performed using the avidinbiotin complex (ABC) method and an automatic autostainer (CODE-ON immune/DNA slide stainer: Biotek solution, Santa Barbara, CA). Formalin-fixed slides were deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 minutes, antigen retrieval for 15 minutes using Biogenex Antigen Retrieval Citra solution in 90°C water bath for 30 minutes. The slides were allowed to cool for 20 minutes before continuing. Slides were then blocked by normal horse serum for 5 minutes at 37°C. The monoclonal antibody was applied overnight in humid medium at room temperature followed by the biotinylated secondary antibody for 15 minutes at 37°C and the ABC complex for 15 minutes at 37°C (Vectastain Elite ABC kit; Vector laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 minutes at room temratureas chromogenic slides was counterstained with haematoxylin, dehydrated, and covered by cover slips. In negative control slides, the same system was applied with replacement of the monoclonal antibody by diluted normal bovine serum. PCNA- immunostaining was performed using polyclonal rabbit-anti-human (A3533 Ig fraction; DAKO, Glostrup, Denmark).

Statistical analysis

Data were presented as the mean ± standard error of mean (SEM).The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 16). Student t- test was performed to assess the significance of differences between groups. Significance at $P<0.05$ was considered statistically significant.

4 Results

Histological results

Examination of sections of control liver showed that the structural unit of the liver is the hepatic lobule which is made up of radiating plates, cords, or strands of hepatocytes forming a network around central vein **(Fig.1)**. Also, Examination of liver section prepared from animals treated with folic acid is identical to that observed in normal control **Fig. (2)**. Sections of livertreated with L-thyroxin showed impaired structural organization of the hepatic lobules. In addition central and portal veins were congested and enlarged with blood and surrounded by leucocytic infiltration. Most of hepatocytes displayed cytoplasmic vacuolation and the nuclei are pyknotic, fatty infiltrations were observed in different parts of the liver section **(Figs. 3 - 6).** Liver sections of L-thyroxin treated with folic acid group showed a good degree of improvement in the hepatocytes. Mild congestion in central and portal vein surrounded by few leucocytic infiltrations were observed (**Figs. 7, 8**).Finally, liver sections of recovered group showed a disturbance of the hepatocytes with hepatocellular vacuolations, congestion in both central and portal veins, surrounded by leucocytic infiltrations, **(Figs. 9 , 10).**

Immunohistochemical results:

1) PCNA immunoreactivity observations:

Examination of liver sections of control group stained by immunohistochemistry showed few expression of PCNA was observed (**Fig. 11**). Negative expression of PCNA in a section treated with folic acid (**Fig. 12**). Strong positive expression of PCNA was detected in the liver section of L-thyroxin group respectively (**Fig. 13**). However; mild positive expressions of PCNA were observed in rat treated with L-thyroxin and folic acid in liver section respectively (**Fig. 14**). Strong positive expression of PCNA was detected in the liver section of recovered group (**Fig. 15**).

2) P53 immunoreactivity observations:

Examination of liver sections in control group and folic acid group stained by immunohistochemistry showed negative expression of P53 (**Fig. 16).** Sections in folic acid group showed faint stain of P53 (**Fig. 17).** Strong expressions of P53 were detected in the liver sections in L-thyroxin group and recovered group **(Fig. 18).** However; positive expressions for P53 were observed in rat treated with L-thyroxin and folic acid in liver sections respectively **(Fig. 19).** Mild expressions of P53 were detected in the liver sections in recovered group **(Fig. 20).**

BCL2 immunoreactivity observations:

Examination of liver sections of control group stained by immunohistochemistry showed a few expression of Bcl-2 (**Fig. 21).** Few expression of Bcl-2 was recorded in a section treated with folic acid (**Fig. 22).** Strong positive expressions of Bcl-2 were detected in liver section of L-thyroxin treated rats **(Fig. 23).** However; mild positive expressions of Bcl-2 were observed in rat treated with L-thyroxin and folic acid in liver section respectively **(Fig. 24).** Strong positive expressions of Bcl-2 were detected in liver section of recovered rats **(Fig. 25).**

Biochemical results:

a.Change in serum ALT and AST

Data in table (**1**) revealed the changes in serum ALT and AST levels in L-thyroxin group were significantly increased when compared with control group. There were significant changes in serum ALT and ASTlevels between L-thyroxin group and rat treated with L-thyroxin and folic acid. Serum ALT and AST levels in recovered group were significantly decreased when compared with L-thyroxin group and rat treated with L-thyroxin and folic acid.

b. Changes in MDA level

Data in **(Fig. 26)** revealed the changes in liver homogenate Malondialdehyde (MDA) there was significant increase in MDA values in the L-thyroxin group as compared to the control group. The MDA values in rat treated with L-thyroxin and folic acid and L-thyroxin group was extremely significant as compared to the control group. Rat treated with L-thyroxin and folic acid reduced the MDA values as compared to recovered group. The MDA value of recovered group was higher than all groups.

c. Changes in nitric oxide level

Data in (**Fig. 27**) revealed the changes in live homogenate nitric oxide values of L-thyroxin group and recovered group showing extreme significance as compared to the control group and rat treated with L-thyroxin and folic acid showed a significant value as compared to the control group. There is no significant difference between results of nitric oxide except L-thyroxin group and recovered group which show significant increase as compared to the other groups.

d. Changes in reduced glutathione level

Data in (**Fig. 28**) revealed the changes in liver homogenate values of reduced glutathione of L-thyroxin group and recovered group was significant as compared to the control group. There is not a big difference between groups. L-thyroxin group showed the lowest value, and the control group showed the highest value.

4 Discussion

Hyperthyroidism is a very common clinical disorder that is associated with abnormalities in many organs, including liver and kidney.

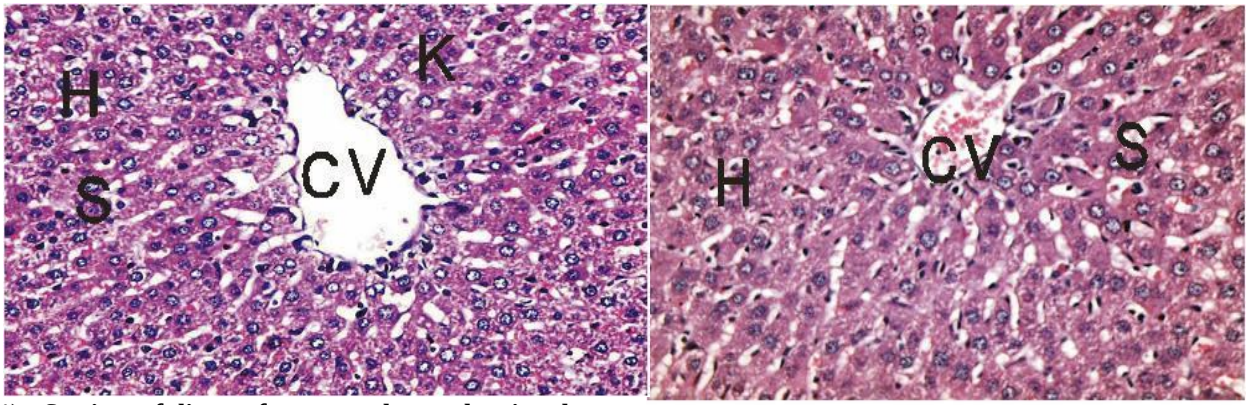


Fig.(1): Section of liver of a control rat showing large polygonal cells with prominent round nuclei and central vein (CV), hepatocytes (H), hepatic sinusoids (S) are arranged in-between the hepatic cords, Kupffer cells (K), (H&E.,X400).
Fig.(2): Section of liver of a rat treated with folic acid showing central vein (CV), hepatocytes (H) and few spaced hepatic sinusoids (S) arranged in-between the hepatic cords, Kupffer cells (K), (H&E.,X400).

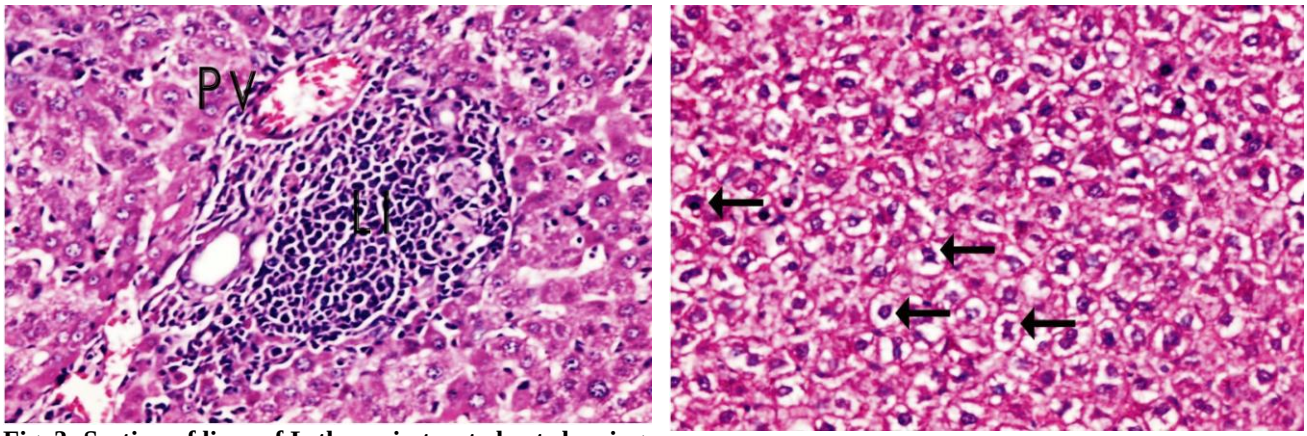


Fig. 3: Section of liver of L-thyroxin treated rat showing congested portal vein (PV) surrounded by leucocytic infiltration (Li), (H&E., X400).
Fig. 4: Section of liver of L-thyroxin treated rat showing cytoplasmic vacuolations of the hepatocytes, the nuclei are pyknotic (arrows), (H&E., X400).

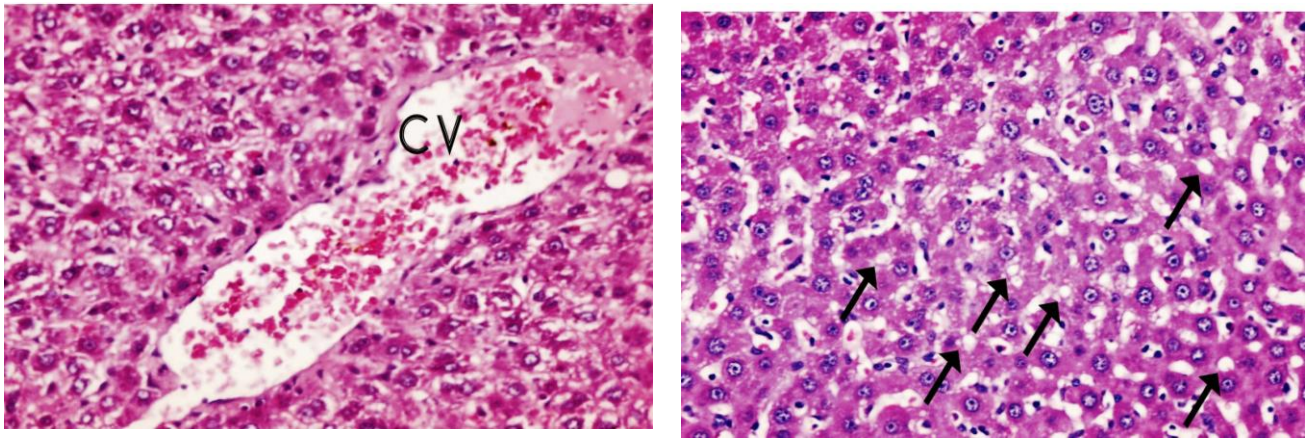
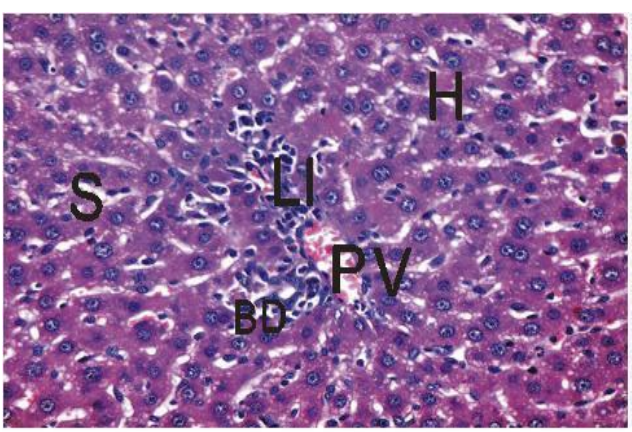


Fig. 5: Section of liver of L-thyroxin treated rat showing enlarged congested central vein, (H&E., X400).

Fig. 6: Section of liver of L-thyroxin treated rat showing fatty infiltrations (arrows), (H&E., X400).



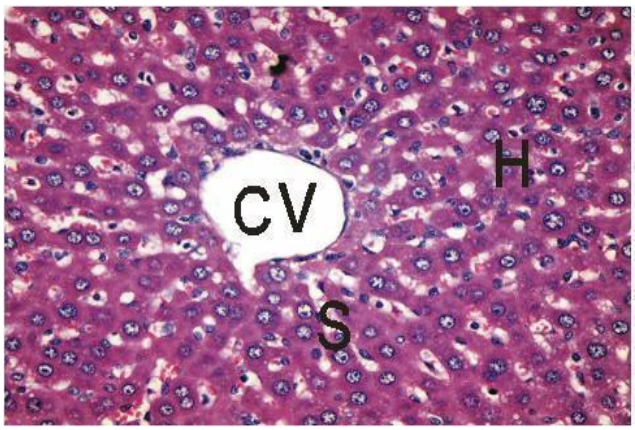
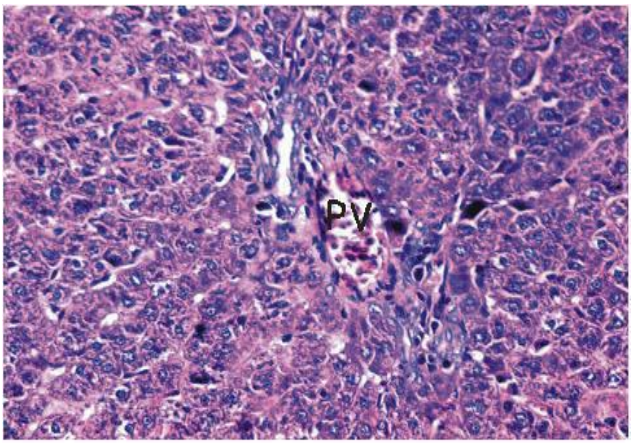


Fig. 7: Section of liver of rat treated with L-thyroxin and folic acid showing an improvement in hepatocytes. leucocytic infiltrations and few congestion in portal veins were observed, (H&E., X400).
Fig. 8: Section of liver of rat treated with L-thyroxin and folic acid showing normal arrangement of the hepatocytes (H), central vein (V) and sinusoids (S), (H&EX400).



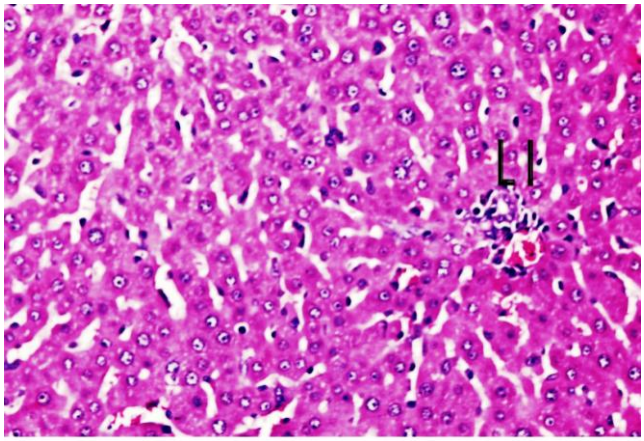
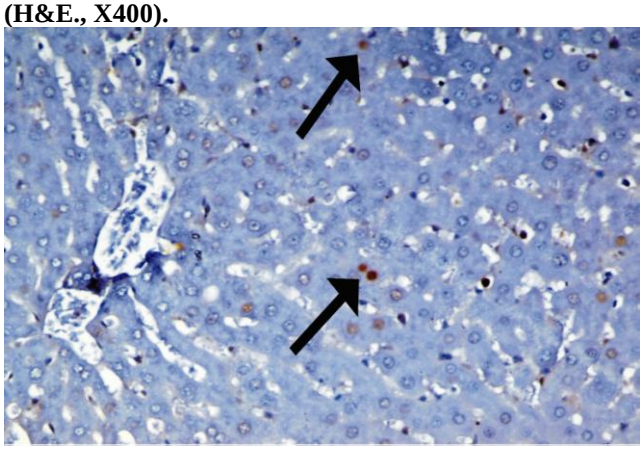


Fig. 9: Section of liver of recovered rat showing congestion of portal vein (PV), (H&E., X400).
Fig. 10: Section of liver of recovered rat showing slight recovery of heptocytes with few leucocytic infiltrations, (H&E., X400).



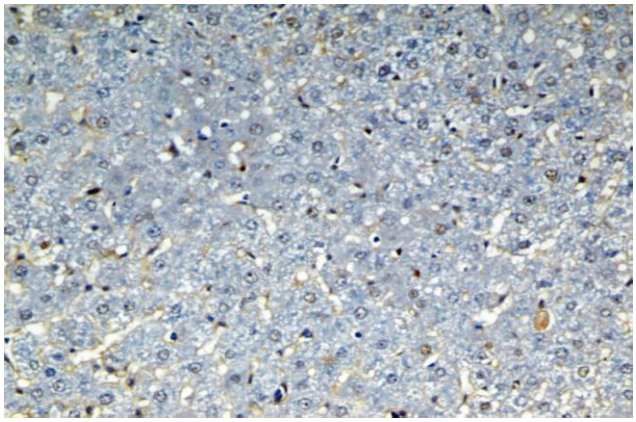
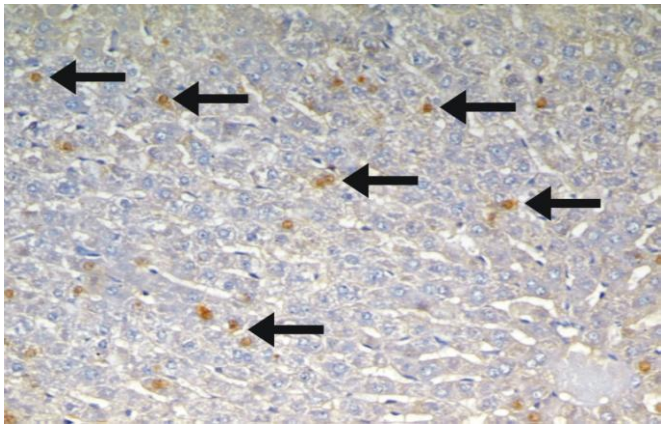


Fig. 11: Section of liver of control rat showing few expression of PCNA, (PCNA immunohistochemical stain, X400).
Fig. 12: Section of liver of folic acid rat showing negative expression of PCNA, (PCNA immunohistochemical stain, X400).



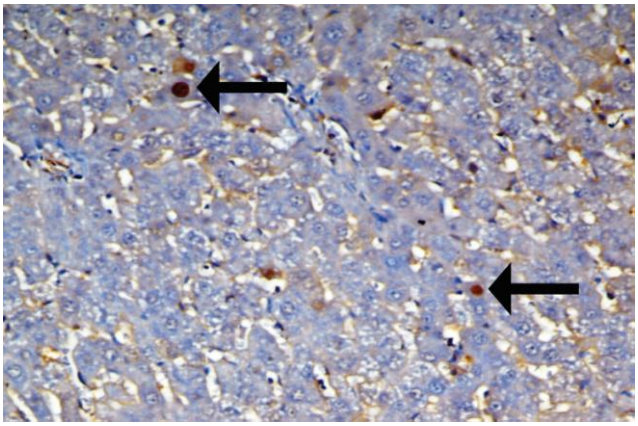
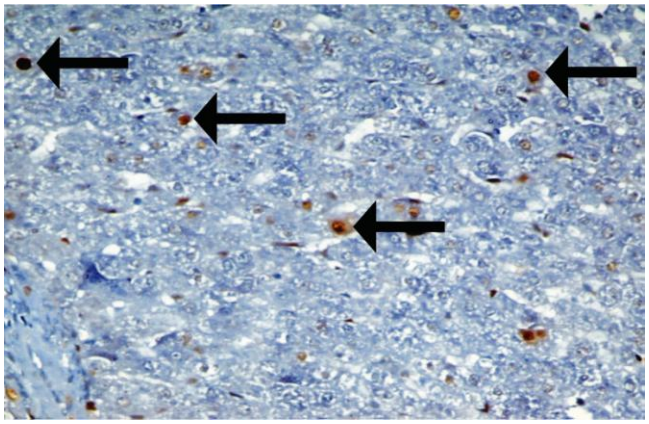


Fig. 13: Section of liver of L-thyroxin rat showing strong positive expression of PCNA, (PCNA immunohistochemical stain, X400).
Fig. 14: Section of liver of rats treated with L-thyroxin and folic acid showing mild positive expression of PCNA, (PCNA immunohistochemical stain, X400).



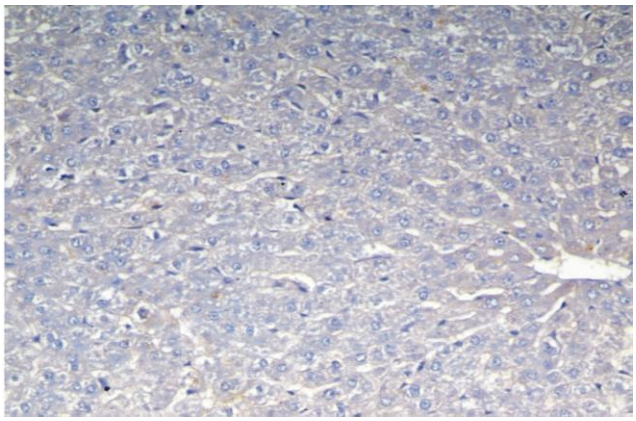
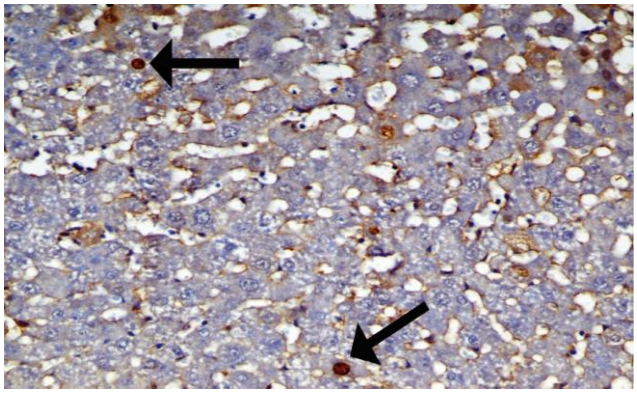


Fig. 15: Section of liver of recovered rat showing strong positive expression of PCNA, (PCNA immunohistochemical stain, X400).
Fig. 16: Section of liver of control rat showing negative expression of P53 in nuclei (arrows), (P53 stain and counter stain in haematoxilin. X400).



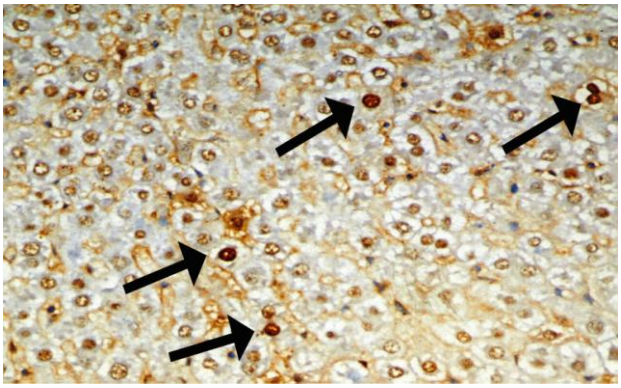
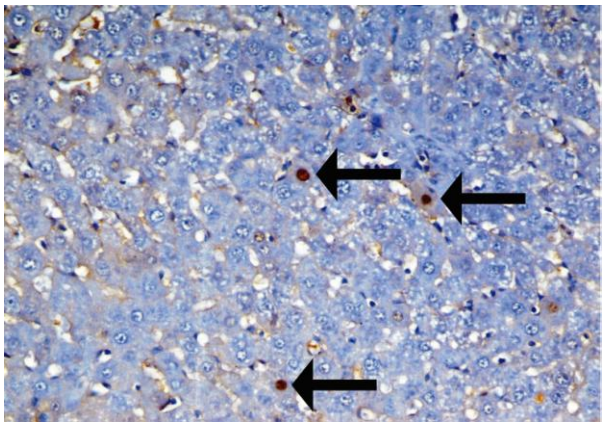


Fig. 17: Section of liver of folic acid rat showing faint stain of P53 in nuclei of hepatocytes, (P53 immunohistochemical stain, X400).
Fig. 18: Section of liver of L-thyroxin treated rat showing strong expression of P53 in most of hepatocytes, (P53 immunohistochemical stain, X400).



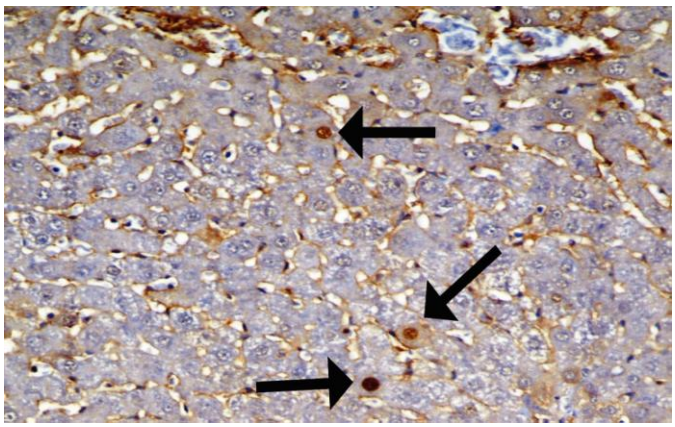
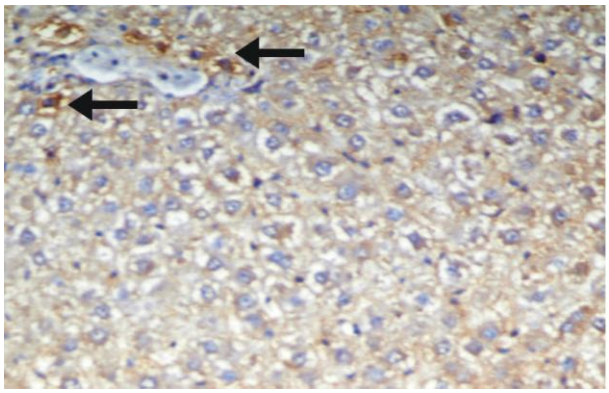


Fig. 19: Section of liver of rat treated with l-thyroxin and folic acid showing weak expression of P53 in most of hepatocytes, (P53 immunohistochemical stain, X400).

Fig. 20: Section of liver of recovered rat showing mild expression of P53 in most of hepatocytes, (P53 immunohistochemical stain, X400).



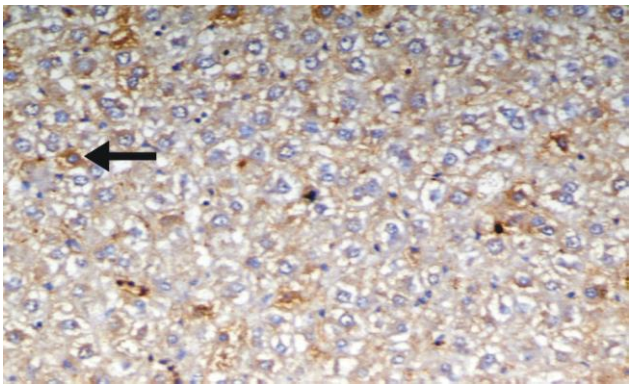
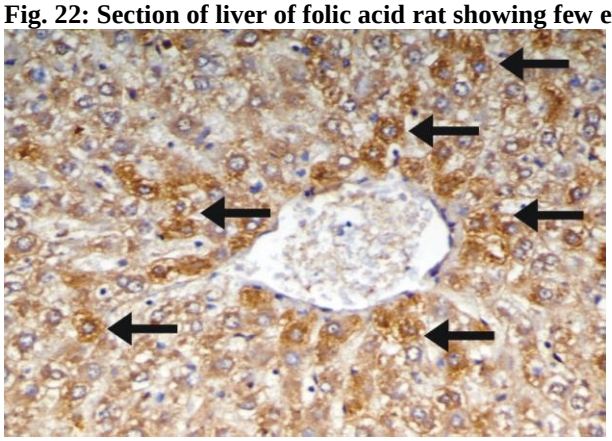


Fig. 21: Section of liver of control rat showing few expression of Bcl-2, (Bcl-2 immunohistochemical stain, X400).

Fig. 22: Section of liver of folic acid rat showing few expression of Bcl-2, (Bcl-2 immunohistochemical stain, X400).



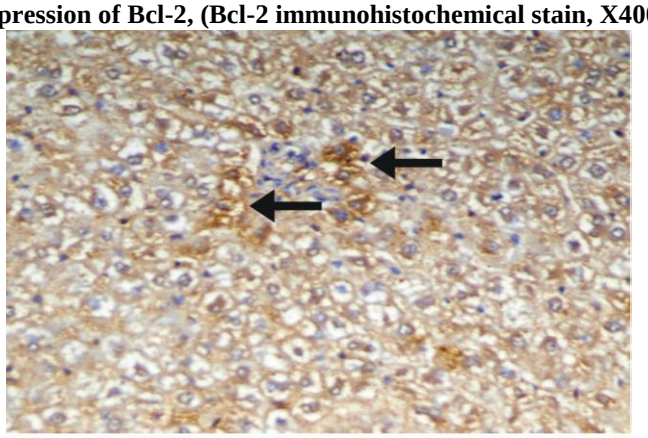


Fig. 23: Section of liver of L-thyroxin rat showing strong positive expression of Bcl-2, (Bcl-2 immunohistochemical stain, X400).

Fig. 24: Section of liver of post treated rat showing few expression of Bcl-2, (Bcl-2 immunohistochemical stain, X400).

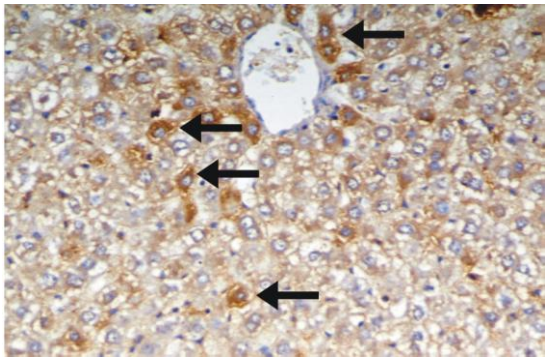


Fig. 25: Section of liver of recovered rat showing strong positive expression of Bcl-2, (Bcl-2 immunohistochemical stain, X400).

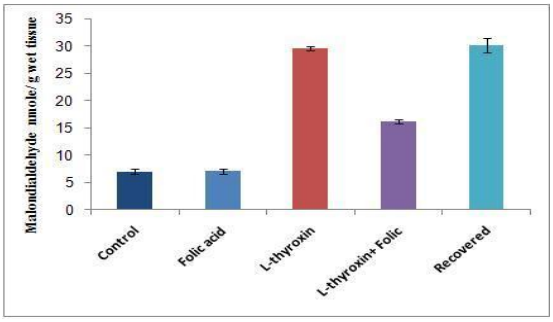


Fig. 26: Changes in liver homogenate MDA level in different groups under study.

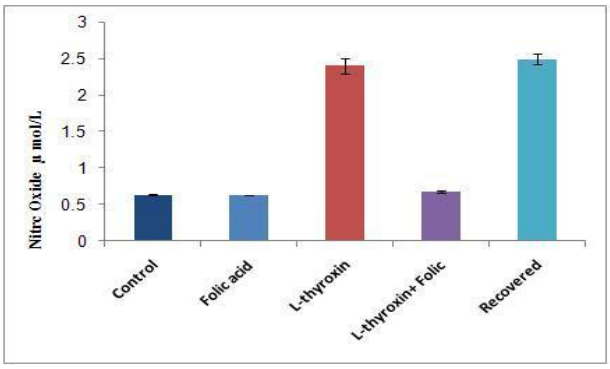


Fig. 27: Changes in liver homogenate nitric oxide level in different groups under study.

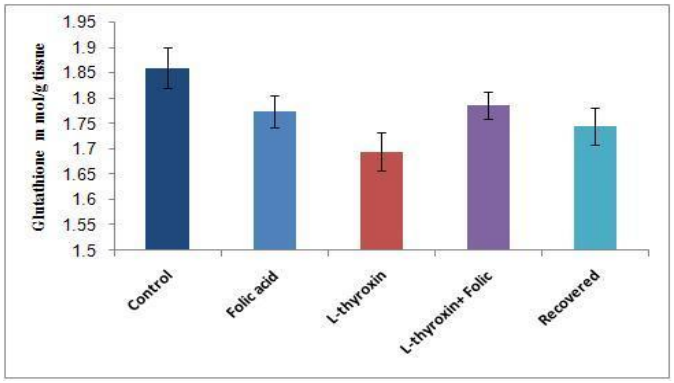


Fig. 28: Changes in liver homogenate GSH level in different groups under study.

Table 1: Changes in serum SGPT (ALT) levels in different groups under study

Groups	SGPT (ALT) (U/L)	SGOT (AST) (U/L)
G1	27.00 ± 1.578	30.40 ± 0.884
G2	26.30 ± 1.506	30.90 ± 0.737
G3	62.50 ± 1.916 ***	66.60 ± 2.587 ****
G4	20.40 ± 0.909 **	25.50 ± 0.763 ***
G5	32.60 ± 1.643	35.20 ± 0.611 ***

Where G1, Control group; G2, folic acid group; G3, L-thyroxin group; G4, rat treated with L-thyroxin and folic acid; G5, recovered group.

The obtained results revealed that treating rats with L-thyroxin induced many histopathological alterations in liver. These alterations include leucocytic infiltrations, congestion of central and portal veins and cytoplasmic vacuolation of hepatocytes with the presence of pyknotic nuclei, in addition to fatty infiltration. These results are in agreement with a number of studies which provided evidence that hyperthyroidism causes an adverse effect on the liver tissue. Steller, (1998) reported that the microscopic examination of the histological sections of the liver of the animal treated with L-thyroxin showed an observed cytoplasmic vacuolization, hemorrhage, and degeneration of the majority of hepatic cell. It was also noted an increase in the number of blood vessels and infiltration of inflammatory cells, which refers to an inflammatory response resulting from the release of lysosomal enzymes and the secretions of degenerative cells, which led to the attraction and migration of these cells into the tissue, which is considered a means of defense in vivo. Lawrence et al., (1991) discussed the pathological changes in different organs in case of hyperthyroidism. Liver of hyperthyroid animals showed vacuolar degeneration and necrosis of hepatocytes. This change may be also as a result of thyrotoxicosis. Rubin, (2001) mentioned that in hyperthyroidism, the hepatocytes showed fatty degeneration and inflammatory cellular infiltration especially in the area of portal tracts. Also, inflammation is a reaction of microcirculation characterized by movement of fluids and leukocytes from the blood into the extra vascular tissue. Moreover accumulation of fats within the hepatocytes could be related to impaired protein synthesis as a result of rER damage and accordingly inhibition of lipoprotein manufacture which is involved in the transport of hepatic triglycerides to extra hepatic tissue and its inhibition results in the accumulation of fats in the cytoplasm of pathophysiological conditions. It is responsible for the deletion of unwanted cells. Apoptosis of individual cells may present a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells (Wang et al., 2003; Tousson et al., 2012, 2014). There are numerous external signals that are involved in the regulation of the apoptosis; P53 is one of the most extensively investigated pathways (Nagatu., 1997; Tousson et al., 2012).

In the present study serum ALT and AST revealed a significant increase in hyperthyroid group when compared with control group. In this concern, Khemichian and Fong, (2011) reported abnormal results of liver function tests in patients with hyperthyroidism and the diagnosis of concomitant, unrelated liver disease difficult until the euthyroid state has been established. . Amin and Hamza, (2005) found that clinical diagnosis of disease and damage to the structural integrity of liver is commonly assessed by monitoring the status of serum AST and ALT activities.
Feng et al. (2000) and Simon-Giavarotti et al. (2002) approved that hyperthyroidism is accompanied with the increase in marked oxidative impact as evidenced by the significant increase in hepatic lipid peroxidation and cell damage markers (AST, ALT, and ALP activities).

Immunohistochemical observations of the liver tissue of hyperthyroid animals showed a significant increase of the apoptotic protein p53 and a significant increase in the anti apoptotic Bc1-2 proteins after L-thyroxin administration. The expression of the nuclear p53 and the incidence of the apoptotic cells were very low in the control liver sections. Apoptosis, or programmed cell death, is a crucial cellular activity in the behavior of mammalian cells in a wide range One of the apoptotic pathways is dominated by Bcl-2 family of proteins. This protein has a role in governing the release of cytochrome C from the mitochondria (Cory and Adams, 2002). So, it is important in protecting the cell against the apoptosis without affecting cell proliferation. When there is an excess of anti-apoptotic proteins, the cells are more resistant to apoptosis (Scorrano and Korsmeyer, 2003; Tousson et al., 2014). The increasing of p53 apoptotic cells and Bcl-2 anti apoptotic cells in the present study revealed the possibility of the apoptosis occurrence after L-thyroxin administration.

Also, immunohistochemical results showed that there is an increase in the expression of PCNA in liver following L-thyroxin administration in comparison to the control group. Similarly, Heron and Rakusan, (1995)

demonstrated that hypothyroid/hyperthyroid endothelial cells of heart showed significantly higher PCNA.

Results of oxidative parameters revealed that there is a significant increase in MDA and NO in liver homogenate of L-thyroxin group as compared to control group, while there is a significant decrease in GSH. These results are consistent with the results reported by Asayama and Kato (1990) who demonstrated increased MDA levels in liver, heart and muscles of rats. Similarly, MDA contents in liver, heart, and muscles rats were largely increased when treated with thyroxin (Venditti et al., 1997). Under these circumstances the free radicals react with lipids, protein, and DNA often causing irreparable damage that can lead to cell death (Beal, 2005). One of the deleterious consequences of oxidative damage is lipid peroxidation, which involves hydrogen abstraction from fatty acids by free radicals such as ˙OH and once initiated is a self propagating process (Benzie and Strain, 1996). Previous studies have suggested that hyperthyroidism increased free radical production and lipid peroxidation levels (Mogulkoc et al., 2006). Seven et al., (1996) mentioned that free radical scavenging enzyme activity can be induced by excessive formation of ROS in experimental hyperthyroidism. Tissue homogenate glutathione levels were decreased in L-thyroxin treated rats as compared to control rats, possibly secondary to increased ROS generation. These results are in agreement with the results obtained in the study of Araujo et al., (2006) who state that the administration of L-thyroxin to rats results in decrease of glutathione concentration in the heart. The cellular glutathione plays an important role as biological antioxidant defense systems, which act as protective mechanisms against oxidative damage. Therefore, the decreased level of glutathione may be due to overproduction of free radicals and increased lipid peroxidation in hyperthyroidism (Asayama et al., 1987).

Treatment of hyperthyroid rats with folic acid showed an improvement in the biochemical, histological and immunohistochemical alterations that induced by L-thyroxin. These results are in agreements with a number of studies that declare the adjuvant role of folic acid. Folic acid has been reported to have an antioxidant power against free radicals responsible for lipid peroxidation (Joshi et al., 2001; Au-Yeung et al., 2006). Racek et al. (2005) reported folate may be considered as an effective antioxidant in patients with hyperhomocysteinemia; this can be a result of decreased production of free radicals due to a reduced level of homocysteine.Tousson et al. (2013) indicated that folic acid had ameliorative effect against cardiac hypertrophy induced by thyroxin and the best results were found in case of using the folic acid as an adjuvant therapy after returning to the euthyroid state.

In conclusion, the current study indicated that hyperthyroidism in male rats was associated with oxidative stress and other biochemical alterations in serum and homogenate of liver. So, treatment of hyperthyroid rats with folic acid improves the oxidative stress and liver functions.

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