

Folic Acid Alleviates Oxidative Stress and Hyperhomocysteinemia Involved in Liver Dysfunction of Hypothyroid Rats

Ehab Tousson^{1,*}, Wafaa Ibrahim³, Afrah F. Salama², Wesam M. Hussein²

¹Department of Zoology, Faculty of Science, Tanta University, Egypt

²Biochemistry Section, Department of Chemistry, Faculty of Science, Tanta University, Egypt

³Department of medical Biochemistry, Faculty of Medicine, Tanta University, Egypt

*Corresponding author: toussonehab@yahoo.com

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Abstract Thyroid hormones are essential for growth and development of the liver. This study evaluated some biochemical alterations in post-pubertal hypothyroidism and its impact on liver functions. 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, 0.05% propylthiouracil-induced hypothyroid rats; group IV, Co-treatment; group V post-treatment). There was a significant decrease in plasma T₃, body weight, fluid and food intakes, folic acid, ALT, total thiol and tFRAP in hypothyroid rats as compared to control group. On the other hand, a significant increase in TSH, relative liver weight, plasma of total homocysteine, serum total protein, AST, total serum bilirubin, cholesterol and tMDA in hypothyroid rats as compared to control group. This reflects hyperhomocysteinemia and oxidative stress associated with hypothyroid state. Folic acid supplemented after restoration of the euthyroid state presented better amelioration over its concurrent supplementation. If confirmed in human beings, our results could propose that folic acid can be used as an adjuvant therapy in hypothyroidism disorders with thyroxin replacement therapy.

Keywords: hypothyroidism, liver, oxidative stress, folic acid, PTU

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1. Introduction

Thyroid hormones has multiple effects on liver function including stimulation of enzymes regulating lipogenesis and lipolysis as well as oxidative processes [1], also it stimulates protein turnover by stimulating both protein synthesis and degradation, in addition to increase gene expression with concomitant increases in protein synthesis and enzyme activity [2]. Thyroid hormones stimulates turnover of glucose, accordingly it has been found that the cycling between glycogenolysis and gluconeogenesis is stimulated by thyroid hormones [3].

Hypothyroidism has been reported to cause mild hyperhomocysteinemia (increased homocysteine concentration in the blood) [4-8]. The mechanism by which hypothyroidism causes mild hyperhomocysteinemia is not clear but altered remethylation is likely to be involved. Additionally, complex changes in folic acid metabolism occur in thyroid dysfunction with increases in tetrahydrofolate and decreases in methyltetrahydrofolate in hypothyroid rat liver [9]. Alteration in thyroid hormone level is also known to modulate functions of several tissues in adult

animals by modulating their antioxidant defenses [4-8,10-13]. Overt Thyroid dysfunctions are frequently associated with abnormalities of biochemical liver tests and in severe forms with histologically evident hepatocellular damage the activity of hepatic lipase is decreased [14]. Hypothyroid animals have decreased metabolic rate with decreased hepatocytes oxygen consumption [15].

Reversible abnormalities of liver function tests are common, although usually mild, in hypothyroidism. In addition, there is a significant decrease in gluconeogenesis [16]. Hypothyroid patients have specific defects in hepatic handling of amino acids resulting in decreased urea nitrogen generation [17].

Hypothyroidism may alter several critical steps in cholesterol and bile acid synthesis [18]. In addition, thyroid hormone modifies lipoprotein metabolism in the liver [19]. It is unclear whether this is a direct thyroid effect on liver enzymes or secondary to altered intestinal handling of cholesterol and bile acids [20].

There is also evidence that hypothyroidism may directly affect the liver structure or function. Hypothyroidism has been associated in a few case reports with cholestatic jaundice attributed to reduced bilirubin and bile excretion. The triad of reduced bilirubin excretion, hypercholesterolemia and hypotonia of the gall bladder

seen in hypothyroidism increases the incidence of gallstones [21].

Folic acid, also known generically as folate is a member of the B-complex family of vitamins, works in concert with vitamin B12. Folic acid functions primarily as a methyl-group donor involved in many important body processes, including DNA synthesis. Therapeutically, folic acid is instrumental in reducing homocysteine levels. Moreover, this vitamin also has antioxidant properties [22]. So, therefore this work aimed to study the effect of hypothyroidism on liver, total plasma homocysteine level and oxidative stress parameters, aid to assess the possible effect of folic acid in reducing these changes.

2. Materials and Methods

The experiments were performed on fifty male albino rats (*Rattus norvegicus*) weighing (120 g \pm 10 g) and of 6-7 week's age. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard diet and water available *ad libitum*. The temperature in the animal room was maintained at 23 \pm 2°C with a relative humidity of 55 \pm 5%. Light was on a 12:12 hr light -dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research. The rats were randomly and equally divided into five groups (10 animals each).

Group 1: (G₁) Control group in which, rats never received any treatment.

Group 2: (G₂) Folic acid group in which, rats received clear water for two week then folic acid (El Nasr Pharmaceutical Chemicals Co.; 0.011 μ mol/g of body weight/day) for four weeks according to Salama et al. [8].

Group 3: (G₃) Hypothyroid rats group in which, rats received 0.05% 6-n-propyl-2-thiouracil (PTU) in drinking water for 6 weeks according to Tousson et al. [11].

Group 4: (G₄) Co-treatment group in which, rats received 0.05% PTU in drinking water and folic acid simultaneously according to Salama et al. [8]. Rats received PTU for six weeks as in hypothyroid rats group. However, folic acid was administered orally for 4 weeks (from 2nd week to 6th week) after evidence of hypothyroidism had been established at the end of the second week.

Group 5: (G₅) Post treatment group in which, rats received 0.05% PTU in drinking water for 6 weeks as in hypothyroid rats group. Additionally, folic acid was administered for another 4 weeks (from 7th week to 10th week) while PTU was withdrawn after the sixth week to return to the euthyroid state.

During the course of treatment, daily fluid consumption, body weight gain, and feed consumption were recorded periodically. At the end of the experimental period, rats were euthanized. Blood samples were individually collected from each rat in heparinized and non heparinized glass tubes to estimate some parameters in plasma and serum. Also liver were quickly removed and weighed after the removal of the surrounding connective tissues carefully and washed in saline and homogenized for assessment of biochemical parameters.

Biochemical investigations: Serum T₃ was assayed by using commercial test supplied by the Diagnostic systems Laboratories (Taxes, USA) according to the method of

Chopra et al. [23]. Serum TSH was assayed by using commercial Kit supplied by Coat-A-Count TSH IRMA (Los Angeles, USA) according to the method of Engall [24]. AST and ALT activities in serum were assayed by using commercial kit that was supplied by Randox (Egypt) according to the method of Rietman and Frankel [25]. Concentration of bilirubin was spectrophotometrically determined using commercial diagnostic kits supplied by Human (German) according to the method of Jendrassik and Gróf [26]. Concentration of total protein was spectrophotometrically determined using commercial diagnostic kits supplied by Diamond (Egypt) according to the method of Bowers and Wong [27]. Cholesterol concentration was assayed by using commercial kit that was supplied by Spinreact (Spain) according to the method of Allain et al. [28].

Blood plasma was analyzed with High-Performance Liquid Chromatography (HPLC) to determine folic acid and total homocysteine (tHcy). The HPLC instrument was Agilent 1200 series HPLC system from Agilent Technologies (USA). Folic acid was determined by HPLC according to the method of Amidzic *et al.* [29]. Total plasma homocysteine was determined by HPLC according to the method of Jayatilleke and Shaw [30].

MDA in liver was estimated by the method of Placer *et al.* [31] using 1,1,3,3-tetramethoxypropane (Fluka Chemical Company) as MDA standard. Ferric reducing ability of liver homogenate which represent total antioxidant capacity (TAC) was [32].

2.1. Statistical Analysis

Data were expressed as mean values \pm SEM and statistical analysis was performed using one-way analysis of variance (ANOVA) to assess significant differences among treatment groups. The criterion for statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS statistical version 16 software package (SPSS[®] Inc, USA).

3. Results

In order to ensure that PTU induced hypothyroidism we determined the serum T₃ and TSH through the dose period. **Table 1** showed that the serum T₃ levels in hypothyroid rats and in co-treated hypothyroid rats with folic acid was significantly lower when compared with the control group; also post treated hypothyroid rats with folic acid were significantly higher when compared with the hypothyroid rats. On the other hand, **Table 1** showed that serum TSH levels in hypothyroid rats and co-treated hypothyroid rats with folic acid were significantly higher than those found in the control group; also post treated hypothyroid rats with folic acid were significantly lower when compared with the hypothyroid rats.

Table 1 showed that, Plasma Hcy in hypothyroid rats were significantly higher when compared with the control group, also treatment of hypothyroid rats with folic acid (during and after inducing hypothyroidism) were insignificantly lower when compared with the hypothyroid rats (**Table 1**). **Table 2** showed that, body weight gain were significantly lower in hypothyroid rats and in hypothyroid rats which co treated with folic when compared with the control and post treated folic acid

groups. Also relative liver weight (RLW) were significantly lower in hypothyroid rats and in co-treated hypothyroid rats with folic acid when compared with the control and post treated hypothyroid rats with folic acid. The fluid intake in hypothyroid rats and in treated

hypothyroid rats with folic acid groups was significantly lower than control group. The food intake in hypothyroid rats and in treated hypothyroid rats with folic acid groups were significantly lower than control (Table 2).

Table 1. The changes in the concentration of plasma T₃, TSH, folic acid and Hcy levels in different groups under study

	G ₁	G ₂	G ₃	G ₄	G ₅
T ₃ (ng/dl)	147±22.36 ^a	148±23.51 ^a	57.67±6.88 ^b	40±0.00 ^b	142.7± 22.11 ^a
TSH (μIU/ml)	0.013±0.003 ^a	0.15±0.123 ^a	3.9±0.513 ^b	5.36±0.27 ^b	0.06±0.025 ^a
tHcy (μM)	1.37±0.05 ^a	1.28±0.04 ^a	2.30±0.03 ^b	2.04±0.08 ^c	1.9±0.03 ^c
Folic acid (μg/ml)	145.64±27.17 ^a	255.50±53.5 ^a	57.75±9.6 ^b	88.38±13.04 ^c	94.4±20.88 ^c

Data are expressed as mean ± S.E.M of five observations. Superscripts of different letters differ significantly (p<0.05) from each other. Significance of differences between means was determined by least significant differences (LSD) at P < 0.05.

Table 2. Changes in body weight, relative liver weight (RLW), food and fluid intake in different groups under study

	G ₁	G ₂	G ₃	G ₄	G ₅
Food intake (g/rat/day)	19.643±0.199 ^a	19.600±0.267 ^a	11.250±0.313 ^b	11.582±0.435 ^b	17.529±0.548 ^b
Fluid intake (ml/rat/day)	39.3 ± 0.62 ^a	47.9 ± 0.86 ^a	19.1 ± 0.46 ^b	17.3 ± 0.88 ^c	17.7 ± 0.63 ^b
Body weight gain (g)	38.4 ± 2.78 ^a	44.3 ± 2.60 ^a	8.3 ± .25 ^b	9.7 ± .70 ^b	40.9 ± 2.41 ^a
RLW* (g/100 g)	3.4±0.1 ^a	3.1±0.3 ^a	3.8±0.1 ^b	3.4±0.1 ^b	3.3±0.21 ^a

Data are expressed as mean ± S.E.M of five observations. Superscripts of different letters differ significantly (p<0.05) from each other. Significance of differences between means was determined by least significant differences (LSD) at P < 0.05. Body weight gain equals (final weight-initial weight)*100/initial weight. Relative liver weight (RLW) equals liver wt. (g) *100/Final body wt.(g)

Table 3. Changes in liver function parameters and cholesterol in different groups under study

	G ₁	G ₂	G ₃	G ₄	G ₅
ALT (U/l)	26.25±4.52 ^a	20.5±1.44 ^b	21.0±3.27 ^b	23.27±4.02 ^b	27.0±1.95 ^a
AST (U/l)	28.33±1.45 ^a	37.0±1.0	44.0±6.5 ^b	54.3±2.33 ^c	55 ±3.0 ^c
T. protein (g/dl)	6.32±0.26 ^a	6.45±0.24 ^a	7.25±0.132 ^b	6.42±0.075 ^a	6.55±0.18 ^a
Albumin (g/dl)	2.63±0.088 ^a	2.73±0.29 ^a	3.2±0.11 ^b	3.36±0.088 ^b	3.6 ±0.1 ^b
T. bilirubin (mg/dl)	0.53±0.067 ^a	0.51±0.037 ^a	0.78±0.078 ^b	0.75±0.074 ^b	0.75±0.09 ^b
D. bilirubin (mg/dl)	0.12±0.01	0.13±0.005	0.15±0.003	0.12±0.006	0.13±0.01
Cholesterol (mg/dl)	95.2±5.24 ^a	103±4.97 ^a	156±4.0	124±4.26	115.5±4.83 ^a

Data are expressed as mean ± S.E.M of five observations. Superscripts of different letters differ significantly (p<0.05) from each other. Significance of differences between means was determined by least significant differences (LSD) at P < 0.05

Table 3 showed that serum ALT showed non significant changes in hypothyroid group when compare with other groups. AST in hypothyroid, co-treatment and post-treatment groups showed significant increase as compared to control group. However, AST showed significant decrease in control group as compared to hypothyroid, co-treatment and post-treatment groups. Serum total protein in hypothyroid group showed significant increase as compared to all other groups. co-treatment and post-treated hypothyroid rats with folic acid showed significant decrease when compared with hypothyroid group (Table 3). Total serum bilirubin in hypothyroid, co-treated and post- treated groups showed non significant increase when compared with control group. Serum cholesterol in hypothyroid and in treated hypothyroid rats with folic acid showed significant increase as compared to control group.

On the other hand, treated hypothyroid rats with folic acid showed significant decrease as compared to hypothyroid group (Table 3).

Table 4 showed that oxidative stress which represent by MDA in liver tissues in hypothyroid rats is significantly higher than those found in the control group; on the other hand, oxidative stress were reduced in hypothyroid groups that treated with folic acid. Also, Table 4 showed that total antioxidant capacity, total thiol and FRAP in liver tissue in hypothyroid group showed significant decrease when compared with the control and folic acid groups, also total antioxidant capacity, total thiol and FRAP in liver tissue in hypothyroid rat groups that treated with folic acid showed significant increase as compared to hypothyroid group.

Table 4. changes in the malondialdehyde (MDA) levels, ferric reducing antioxidant power (FRAP) and total thiol in liver tissue of different groups under study

	G ₁	G ₂	G ₃	G ₄	G ₅
tMDA (nmol/g tissue)	77.16±2.53 ^a	64.07±2.65 ^a	128.04±5.3 ^b	67.86±1.98 ^a	65.6±2.02 ^a
tFRAP (μmol Fe ²⁺ /g tissue)	2.13 ± 0.12 ^a	2.02 ± 0.06 ^a	1.39 ± 0.03 ^b	1.54 ± 0.16 ^b	2.85 ± 0.07 ^c
tTAC (m mol/g tissue)	10.14±0.325 ^a	10.43±0.09	9.83±0.16 ^b	9.97±0.196 ^a	9.91±0.06 ^a
Total thiol (m mol/g tissue)	6.1±0.52	7.28±0.71	5.1±0.18	5.23±0.56	5.17±0.07

Data are expressed as mean ± S.E.M of five observations. Superscripts of different letters differ significantly (p<0.05) from each other. Significance of differences between means was determined by least significant differences (LSD) at P < 0.05. NO_x: plasma total NO metabolites (NO₂+NO₃).

Table 5. Correlation coefficient (r) of T₃, tHcy, folic acid and liver MDA with liver FRAP, and liver total thiol in all groups under study

Parameter	T ₃	tHcy	Folic acid	Liver MDA
Liver MDA	-0.640	0.256	-0.176	----
Liver FRAP	0.475	-0.611*	0.255	-0.638*
Liver total thiol	0.034	-0.225	0.201	-0.029

** . Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

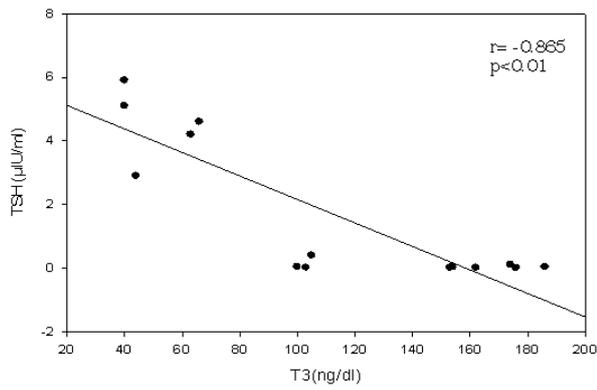


Figure 1. Correlation of serum total triiodothyronine (T₃) with thyroid stimulating hormone (TSH) in different studied groups

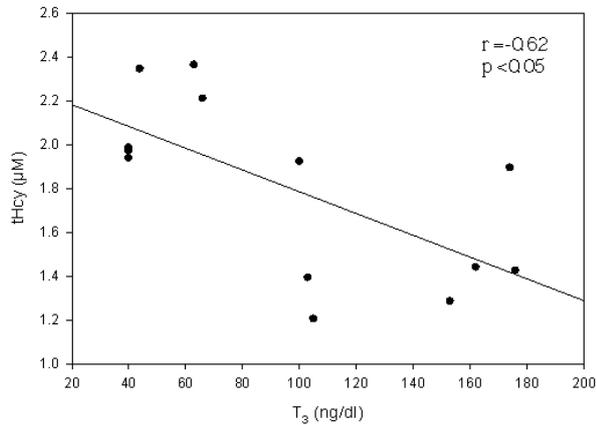


Figure 2. Correlation of T₃ with tHcy in different studied groups

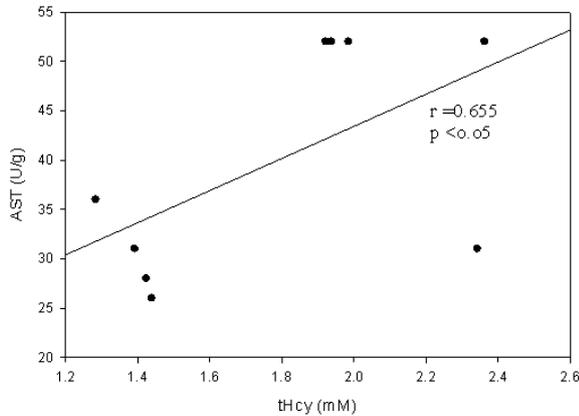


Figure 3. Correlation of tHcy with AST different in different studied groups

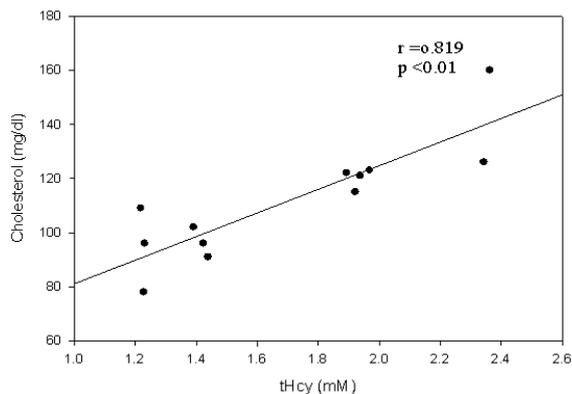


Figure 4. Correlation of tHcy with cholesterol in different studied groups

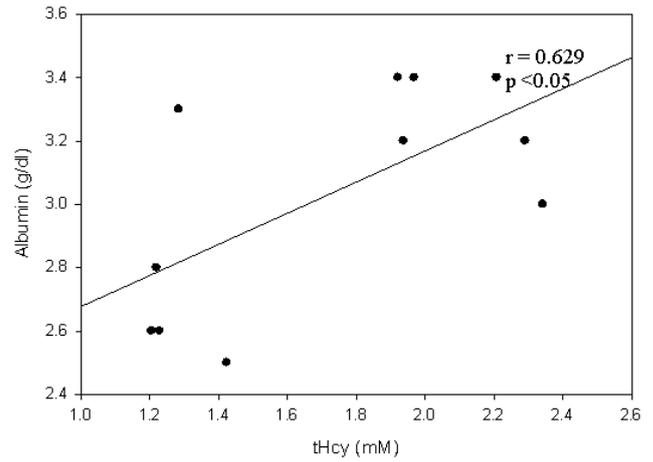


Figure 5. Correlation of plasma total homocysteine (tHcy) with albumin in different studied groups

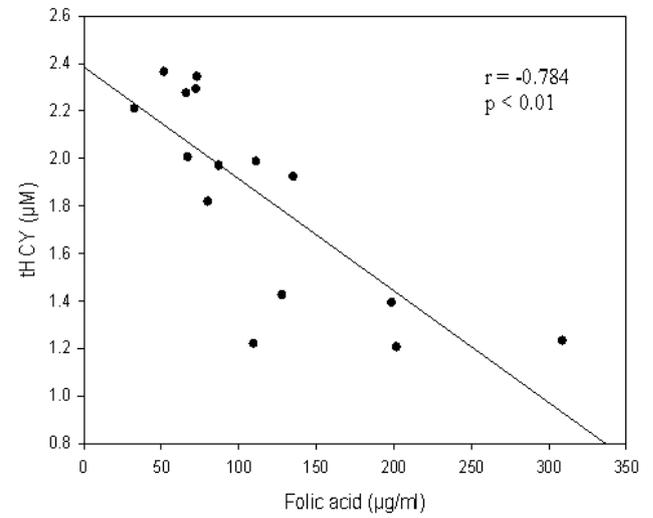


Figure 6. Correlation of plasma folic acid with plasma total homocysteine (tHcy) in different studied groups

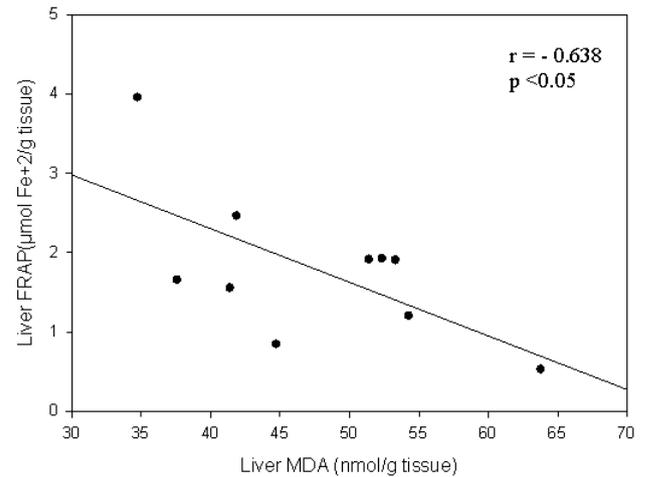


Figure 7. Correlation of liver MDA with liver FRAP in different studied groups

Pearson correlation coefficient of different studied parameters in different studied groups:

In Table 5 and Figure 1- Figure 7, a significant negative correlation was detected between total T₃ with TSH, tHcy in all groups. On the other hand, a significant positive correlation was detected between tHcy with AST, cholesterol and albumin in all groups. Folic acid was

positive correlated with FRAP and total thiol in liver, however a negative correlation with MDA in liver tissues. However negative significant correlations were detected between folic acid with tHcy. In addition to liver MDA had significant negative correlation with liver FRAP.

4. Discussion

The present study was designed to investigate the effect of hypothyroidism on rat liver structure and functions and the possible ameliorating effect of folic acid in treatment. In order to achieve this target we made a hypothyroid state by using a reversible goitrogen (PTU) that known to decrease the conversion of peripheral T_4 to T_3 and thereby reduce serum T_3 concentration [4,5,8,11,33]. Hypothyroid state was documented by changes in T_3 and TSH. The overall changes in the thyroid hormones profile reflect the observed decline in body weight gain. The decrease in body weight gain was associated with a reduction in food and fluid consumption in groups of hypothyroidism. These findings are compatible with the results of other studies [11].

Folic acid exerts an important role over homocysteine catabolism by methyl group donation in remethylation pathway to methionine [34]. In the present study serum Hcy in hypothyroid rats were significantly higher than those found in the control, also Hcy in hypothyroid rats which treated with folic acid during and after inducing hypothyroidism were insignificantly lower than those found in the hypothyroid rats. This finding is in line with that of Diekman et al. [35] and Ibrahim et al. [4] who reported that plasma Hcy concentrations increased in hypothyroidism and decreased in hyperthyroidism the mechanism of thyroid-induced changes in homocysteine metabolism is currently unknown. Adrees et al. [36] who observed that total Hcy levels were elevated in patients with subclinical and overt hypothyroidism. The explanation of this effect could be attributed to the reduced glomerular filtration rate in hypothyroidism which is linked to impaired renal Hcy clearance and hyperhomocysteinaemia [37]. Alternatively, changes in the concentrations or biologic activity of vitamin B12 or B6, and/or folate may occur in response to altered thyroid homeostasis [38].

Liver is a major target organ for thyroid hormone with important biological and medical implications [39]. 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. In the present study AST elevation may be attributed to myopathies which are usually associated with hypothyroidism [40]. It has been well established that thyroid hormone affects cholesterol concentration, hepatic metabolism and cholesterol synthesis [41]. In the present study, serum cholesterol in hypothyroid, co-treatment and post-treatment groups showed significant increase as compared to control group. However co-treatment and post-treatment groups showed significant decrease as compared to hypothyroid group. Hypercholesterolemia might have resulted from increased mobilization of body fat reserves as a result of increased thyrotrophic hormone level in induced hypothyroidism [42]. Moreover, hypercholesterolemia and hyperlipidemia and poor body weight have been suggested as excellent indicators of

suspected hypothyroidism [43]. Thyroid hormones are predominantly considered as hormones involved in the catabolic processes of lipids [44]. Variations in the serum total cholesterol, HDL and LDL-VLDL cholesterol, and total cholesterol in the liver and muscle accounts for the variation in the activity of thyroid hormones on cholesterol biosynthesis and degradative pathways. The most noticeable fact is that hypothyroidism results in the accumulation of cholesterol in the liver and thyroid hormone treatment reduces it [44]. There are relationships between hypothyroidism and hypercholesterolemia and hyperhomocysteinemia [45]. Karmin et al. [46] suggested that homocysteine effects cholesterol production and secretion.

Total serum bilirubin in hypothyroid, co-treatment and post-treatment groups showed significant increase as compared to control and folic acid groups. The increased bilirubin levels observed in the hypothyroid group in the present study might be explained by the findings from earlier observational studies, which proposed that the activity of bilirubin UDP glucuronyl transferase is decreased, resulting in a reduction in bilirubin excretion [40], thyroid function affects bile flow and composition so bilirubin excretion decreases in hypothyroid and increases in hyperthyroid rats [47]. The liver synthesizes a number of plasma proteins so if liver was affected by hypothyroidism; these proteins would be altered. Total protein in serum of hypothyroid rats showed significant increase as compared to all other groups. Co-treatment and post-treatment groups showed significant decrease as compared to hypothyroid group. The resultant increase in inflammatory proteins and immunoglobulins may account for marginally raised serum proteins observed in hypothyroid subjects [48].

It has been well documented that thyroid dysfunction increases lipid peroxidation reactions (evidenced by the elevated MDA levels) and reactive oxygen species [49], and these match with the present study as there was significantly higher of MDA in liver homogenate of hypothyroid group when compared to the control and folic acid groups. The significant decrease in total thiol of liver homogenate in hypothyroid group compared to control group reflects increasing oxidative stress and reduction of antioxidants effectiveness with hypothyroidism [50]. The significant increase in total thiol of liver homogenate in co-treatment as compared to hypothyroid group was explained by the role of folic acid in restoring FRAP without restoration of euthyroid state as presented here. However liver homogenate of post-treatment show significant increase in total thiol as compared to hypothyroid group, this was explained by the role of folic acid and normal thyroid hormone. This finding confirms what previously mentioned that thyroid hormones regulate protein, and antioxidant enzymes synthesis and degradation [4,13].

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