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Folic acid alleviates oxidative stress and hyperhomocysteinemia involved in testicular dysfunction of hypothyroid rats

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ABSTRACT

Although there is general agreement that thyroid hormone is an important hormonal regulator of testis physiology during development period, its role in the post-pubertal and adult testes is still controversial. Furthermore, most experimental studies to date have focused on thyroid hormone effects on the developing testes and only limited data are available on its role in spermatogenesis. This study evaluated some biochemical alterations in post-pubertal hypothyroidism and its impact on testicular function. 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). Plasma total homocysteine, total NO metabolites, malondialdehyde and GSSG/GSH ratio quantified by HPLC significantly ($P < 0.05$) increased in hypothyroid rats as compared to controls. These biochemical alterations at least in part disrupted spermatogenesis in these experimental models. Folic acid supplemented after restoration of the euthyroid state (group V) presented better amelioration to spermatogenesis over its concurrent supplementation (group IV). This postulates an indirect negative impact of post-pubertal hypothyroidism on testicular function through development of these alterations. This is plus the observed role of folic acid supplementation in enhancing spermatogenesis, boosting sperm concentration and building up the antioxidant status against the oxidants in the present study. 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.

1. Introduction

In mammals, altered thyroid status is known to adversely affect many organs and tissues. Nevertheless, for many years, the impact of thyroid disorders on male reproduction remained controversial. Early studies in the 1950's demonstrated that testes were essentially independent of thyroid hormone effects. However, in the past two decades, clinical studies have demonstrated that thyroid hormone plays an important role in testicular development and function [47].

It is now established that T_3 regulates the maturation and growth of testis, controlling Sertoli cell and Leydig cell proliferation and differentiation during testicular development in rats and other mammal species [42]. These data, in conjunction to the findings that thyroid hormone receptors and iodothyronine deiodinases are present in human and rat testes from neonatal to adult life [2,42], confirm that thyroid hormone plays a key role in testicular development.

On the other hand, although there is general agreement that thyroid hormone is an important hormonal regulator of testis physiology during development period, its role in the post-pubertal and adult testes is still controversial. Furthermore, most experimental studies to date have focused on thyroid hormone effects on the developing testes and only limited data are available on its role in spermatogenesis [47].

Propylthiouracil (PTU) is known to inhibit thyroid hormone synthesis and conversion of peripheral T_4 to T_3 and thereby reduces serum T_3 concentration. PTU is also used in treating hyperthyroid conditions like Graves' disease. It has been linked with certain side effects such as transient leukopenia and vasculitis [5]. Thus, chemical induction of hypothyroid state by antithyroid drugs as PTU has been widely established to investigate the role of thyroid hormones in testicular physiology [38].

Hypothyroidism has been reported to induce mild hyperhomocysteinemia and endothelial dysfunction through reduced endothelial NO bioavailability [46]. However, the impact of hyperhomocysteinemia and endothelial dysfunction on testicular

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function is unclear. Besides, regulatory role of thyroid hormone in testicular physiology is well established [38]; however, its effect on testicular antioxidant defense system is still under study [47].

Folic acid has been reported to have an antioxidant power against ROS and an alleviating role in hyperhomocysteinemia and the associated endothelial dysfunction [31]. Also, progressive folate deficiency was suggested to develop with hypothyroidism [13]. This deficiency may be responsible for reduced sperm concentration [48]. Supporting this assumption, a high affinity folate binding protein has been identified in human semen and prostate gland [18]. This finding supports the connection between folate status and male reproductive function. This further illustrates the need for an intact folate cycle to maintain normal spermatogenesis and the positive effect of folic acid on sperm parameters [15]. It is, however, suggested, that changes in folate level may be responsible for the increased serum Hcy level in patients with hypothyroidism [26].

The present study represented a contribution to understand the effect of low thyroid hormone status on total plasma homocysteine level and oxidative stress parameters. Additionally, the impact of these biomarkers on testicular function in PTU-induced hypothyroidism at the post-pubertal stage of male rats was investigated. It also aimed to elucidate the role of folic acid supplementation in enhancing spermatogenesis, boosting sperm concentration and building up the antioxidant status as a concurrent treatment with hypothyroidism and as a post-treatment after restoration of the euthyroid state.

2. Materials and methods

The experiment was performed on 50 male albino rats (*Rattus norvigicus*) weighing 120 g (±10) and of 6–7 week's age. They were obtained from our laboratory farms, Zoology Department, Faculty of Science, Tanta University, Egypt. The rats were kept in the laboratory for 1 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 available *ad libitum*. The temperature in the animal room was maintained at 23 ± 2 °C with a relative humidity of 55 ± 5%. Light was on a 12:12 h light–dark cycle. Fluid and food intake were monitored daily for the group of animals in each cage and divided by the number of each cage animals. Also, body mass measurement was recorded weekly for each animal under experiment. All the experiments were done in compliance with the guiding principles in the care and use of laboratory animals. The rats were equally divided into five groups (10 animals each).

Group I: Control group in which animals never received any treatment (euthyroid).

Group II: Folic acid group in which animals received folic acid (El Nasr Pharmaceutical Chemicals Co.; 0.011 μmol/g body weight/day) only for 4 weeks (from 2nd week to 6th week after the experiment start) orally by a stomach tube [27].

Group III: Hypothyroid group in which a chemical experimental rat model of hypothyroidism that mimics hypothyroidism in humans has been developed. Rats received 0.05% 6-*n*-propyl -2-thiouracil (PTU; Thyrocil®) in drinking water for 6 weeks [38] to cover a complete spermatogenic cycle in rats [11].

Group IV: Co-treatment group in which animals received 0.05% PTU in drinking water and folic acid (0.011 μmol/g body weight/day) concurrently according to Matte et al. [27]. The dose period of PTU was 6 weeks as in hypothyroid rats group. However, folic acid was administered orally by a stomach tube for 4 weeks form the second to 6th week after evidence of hypothyroidism had been established at the end of the 2nd week after the experiment start.

Group V: Post-treatment group in which animals received 0.05% PTU in drinking water for 6 weeks as in hypothyroid group. Additionally, folic acid was administered for another 4 weeks (from 7th week to 10th week after the experiment start) while PTU was withdrawn after the 6th week to establish the euthyroid state [38].

At the end of the experimental period, rats from each group were euthanized with intravenous injection with sodium pentobarbital and subjected to a complete necropsy after 10–12 h of fasting. Testes and epididymides were removed, carefully cleaned from adhering connective tissue in cold saline, weighed and quickly stored at −20 °C until analysis.

Blood samples were individually collected from each rat and divided into two parts. The first part was collected in non heparinized glass tubes to estimate serum parameters. Serum was separated by centrifugation at 3000 rpm for 15 min. The collected serum was stored at −18 °C until analysis. The second part was transferred to EDTA-containing glass tubes to obtain blood plasma. Plasma samples were subjected directly to high performance liquid chromatography (HPLC) analysis. Plasma samples were deproteinized by 75% aqueous HPLC grade methanol in a ratio 1:4 (plasma: methanol) v/v then centrifuged at 3000 rpm for 5 min at 4 °C and the supernatants were separated and used for HPLC application.

Serum was analyzed to determine the T_3 and TSH levels. Determination of serum total triiodothyronine (T_3) [50] and thyroid stimulating hormone (TSH) [51] was carried out by solid phase enzyme-linked immunosorbent assay using Biocheck kit, Inc. (USA). The HPLC instrument was Agilent 1200 series HPLC system from Agilent Technologies (USA). HCy, GSH and GSSG were determined by HPLC using the method of Jayatilleke and Shaw [20]. Nitrites and nitrates were determined according to the method of Papadoyannis et al. [34] by HPLC. Total plasma malondialdehyde (MDA) was determined by HPLC according to the method of Karatas et al. [23] and Karatepe [24]. Testosterone concentration was estimated by HPLC as described by Gonzalo-Lumbreras et al. [16].

Testicular homogenate (10%; w/v) was prepared in ice-cold 0.067 M phosphate buffer (pH 7) then, the homogenate was centrifuged at 3000 r.p.m for 10 min. at 4 °C. The resulting supernatant was used to determine the testicular total antioxidant capacity (TAC) and MDA content. TAC or ferric reducing antioxidant power (FRAP) was determined according to Benzie and Strain [1]. The method measures the ferric reducing ability of testicular homogenate. Thiobarbituric acid reactive substance (TBARS) level or malondialdehyde (MDA) in the testicular homogenate was estimated by the method of Mesbah et al. [29].

By laparotomy, the left and right caudal parts of the epididymis were carefully separated from the testes, finely minced in 5 ml of Hanks' buffered salt medium, and incubated at room temperature for 15 min to provide the migration of all spermatozoa from epididymal tissue to fluid [4]. The diluted sperm suspension (10 ml) was transferred to the hemocytometer (Improved Neubauer, Weber, UK), and the settled sperm were counted with a light microscope at 400× magnification (million/ml). Then, the sperm count was calculated relative to the epididymal wt. (sperm/g). The motility assay was conducted by observing the sperm suspension on a slide glass at 37 °C. The percentage of motile spermatozoa was determined by counting more than 200 spermatozoa randomly in 10 selected fields under a light microscope (Olympus microscope), and the mean number of motile sperm × "100/total number of sperms" was calculated [4].

Results were analyzed using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) tests to compare between different groups. Data were presented as means ± SEM. *P* values less than 0.05 were considered significant. Pearson correlation coefficient (*r*): the reliability of an estimate depends on the relationship between two variables and measure

Table 1
Food intake (g/rat/day), fluid intake (ml/rat/day), increase rate of body weight per week (IRBW; %), relative testes weight (RTW; g/100 g) and relative epididymides weight (REW; g/100 g) in different groups under study.

	Group I	Group II	Group III	Group IV	Group V	
					6 W	4 W
Food intake	15.6 ± 0.37[a]	16.3 ± 0.28[a]	9.4 ± 0.41[b]	12.0 ± 0.87[c]	9.0 ± 0.34[b]	16.6 ± 0.59[a]
Fluid intake	37.5 ± 4.8[a]	37.7 ± 4.2[a]	17.7 ± 1.2[b]	17.6 ± 1.3[b]	16.8 ± 1.0[b]	37.8 ± 4.8[a]
IRBW	5.79 ± 0.40[a]	7.42 ± 0.47[b]	2.25 ± 0.30[c]	3.08 ± 0.40[c]	2.65 ± 0.22[c]	5.95 ± 0.25[a]
RTW	1.547 ± 0.045[a]	1.43 ± 0.1235[a]	1.42 ± 0.118[a]	1.66 ± 0.16[a]	1.67 ± 0.093[a]	
REW	0.343 ± 0.046[a]	0.466 ± 0.048[a]	0.359 ± 0.048[a]	0.445 ± 0.047[a]	0.624 ± 0.038[b]	

Data are expressed as means ± SEM of 10 observations. Superscripts of different letters differ significantly ($P < 0.05$) from each other. However the superscripts of the same letter differ non significantly ($p > 0.05$). Significance of differences between means was determined by least significant differences (LSD) at $P < 0.05$. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment; 6 W: in the initial 6 weeks, 4 W: in the extra 4 weeks).

Table 2
Triiodothyronine (T_3; ng/dl), thyroid stimulating hormone (TSH; μIU/ml), total plasma homocysteine (tHcy; μmol/l) and plasma total nitric oxide metabolites (tNO_x; μmol/l) levels in different groups under study.

	Group I	Group II	Group III	Group IV	Group V
T_3	155.8 ± 13.57[a]	156.6 ± 14.86[a]	56 ± 4.93[b]	40.4 ± 0.81[b]	151.2 ± 13.56[a]
TSH	0.072 ± 0.0086[a]	0.051 ± 0.0123[a]	3.780 ± 0.3470[b]	4.180 ± 0.2354[b]	0.050 ± 0.0152[a]
tHcy	1.369 ± 0.05[a]	1.277 ± 0.04[a]	2.301 ± 0.03[b]	2.041 ± 0.08[c]	1.910 ± 0.03[c]
tNO_x	31.404 ± 1.66[a]	38.611 ± 2.02[a,c]	52.237 ± 3.15[b]	42.289 ± 5.10[c]	33.684 ± 1.60[a]

Data are expressed as means ± SEM of five observations. Superscripts of different letters differ significantly ($P < 0.05$) from each other. However the superscripts of the same letter differ non significantly ($p > 0.05$). Significance of differences between means was determined by least significant differences (LSD) at $P < 0.05$. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment).

Table 3
Plasma malondialdehyde (pMDA; nmol/l), testicular malondialdehyde (tMDA; nmol/g), plasma GSSG/GSH raio (pGSSG/GSH) and testicular ferric reducing antioxidant power (tFRAP; μmol Fe^{+2}/g) levels in different groups under study.

	Group I	Group II	Group III	Group IV	Group V
pMDA	37.686 ± 0.83[a]	27.291 ± 3.25[a]	309.829 ± 10.32[b]	130.943 ± 10.16[c]	218.252 ± 8.39[d]
tMDA	47.429 ± 4.79[a]	49.714 ± 7.11[a,c]	130.000 ± 9.90[b]	72.571 ± 7.92[a,c]	73.143 ± 11.48[c]
pGSSG/GSH	0.506 ± 0.014[a]	0.612 ± 0.022[b]	0.579 ± 0.009[b]	0.603 ± 0.021[b]	0.612 ± 0.022[b]
tFRAP	1.67 ± 0.19[a]	1.29 ± 0.23[a,b]	0.93 ± 0.07[b]	1.37 ± 0.23[a,b]	1.16 ± 0.07[a,b]

Data are expressed as means ± SEM of five observations. Superscripts of different letters differ significantly ($P < 0.05$) from each other. However the superscripts of the same letter differ non significantly ($p > 0.05$). Significance of differences between means was determined by least significant differences (LSD) at $P < 0.05$. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment).

of this closeness is such a measure, commonly symbolized as "*r*". All statistical analyses were performed using SPSS statistical version 16 software package (SPSS® Inc., USA).

3. Results

Table 1 showed significant decrease ($P < 0.05$) in food intake, fluid intake and increase rate of body weight per week in hypothyroid group (group III) and initial 6 weeks of post-treatment (group V) as compared to control (group I). However, restoration of euthyroid state with folic acid supplementation as in extra 4 weeks of post-treatment (group V) normalized it. On the other hand, relative testes and epididymides weight showed non significant change in different study groups. However, relative epididymides weight showed significant ($P < 0.05$) increase in post-treatment (group V) when compared to control (group I).

Serum triiodothyronine (T_3) and thyroid stimulating hormone (TSH) levels showed significant ($P < 0.05$) decrease and increase respectively in hypothyroid and co-treatment groups (group III and IV) as compared to control and folic acid groups (group I and II). Meanwhile, there was non significant change in T_3 and TSH levels in folic acid and post-treatment groups (group II and V) as compared to control group (Table 2).

Plasma levels of total homocysteine (tHcy) and total NO metabolites (NO_x) showed significant increase in hypothyroid group as compared to control and folic acid groups (group I and II). In comparison to hypothyroid group, plasma levels of tHcy and total NO_x showed significant decrease in co-treatment group. Besides, in post-treatment group, while plasma levels of total NO_x showed non significant change, plasma level of tHcy showed significant increase as compared to control and folic acid groups. In comparison to hypothyroid group, plasma levels of tHcy and total NO_x showed significant decrease in post-treatment group. In comparison to co-treatment group, while plasma levels of total NO_x showed significant decrease, plasma levels of tHcy showed non significant change in post-treatment group (Table 2).

In Table 3, there was significant increase in plasma and testicular MDA levels in hypothyroid group as compared to control and folic acid groups. On the other hand, plasma and testicular MDA levels showed significant and non significant increases respectively in co-treatment group as compared to control and folic acid groups. In comparison to hypothyroid group, plasma and testicular MDA levels showed significant decrease in co-treatment group. Moreover, plasma and testicular MDA levels showed significant increase in post-treatment group as compared to control and folic acid groups except for testicular MDA level which showed non significant change as compared to folic acid group. Meanwhile, plasma and testicular MDA levels showed significant decrease in post-treatment group as compared to hypothyroid group. In comparison to co-treatment group, plasma and testicular MDA levels showed significant and non significant increase respectively in post-treatment group.

Table 4
Plasma testosterone (ng/ml), sperm count (No./g epididymis × 10^6) and sperm motility (%) in different groups under study.

	Group I	Group II	Group III	Group IV	Group V
Testosterone	1.613 ± 0.40[a]	1.235 ± 0.11[a]	0.711 ± 0.07[b]	0.631 ± 0.10[b]	0.560 ± 0.11[b]
Sperm count	151 ± 4[a]	166 ± 6[a]	115 ± 6[b]	169 ± 5[a]	215 ± 9[c]
Sperm motility	73 ± 1.3[a]	84 ± 1.9[b]	29 ± 1.1[c]	65 ± 1.4[d]	71 ± 1.5[a]

Data are expressed as means ± SEM of five observations. Superscripts of different letters differ significantly ($P < 0.05$) from each other. However the superscripts of the same letter differ non significantly ($p > 0.05$). Significance of differences between means was determined by least significant differences (LSD) at $P < 0.05$. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment).

Table 5
Correlation coefficient (r) of T_3, tHcy and tNO_x with T_3, tHcy, tNO_x and pMDA in different studied groups.

Parameter	T_3	tHcy	tNO_x	pMDA
T_3	–	−0.708**	−0.663**	−0.496*
tHcy	−0.708**	–	0.539**	0.880**
tNO_x	−0.663**	0.539**	–	0.506**

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 6
Correlation coefficient (r) of T_3, tHcy, tNO_x, pMDA and tMDA with tMDA, FRAP, testosterone and sperm count in different studied groups.

Parameter	tMDA	FRAP	Testosterone	Sperm count
T_3	−0.575**	0.287	0.315	0.339
tHcy	0.739**	−0.280	−0.631**	−0.191
tNO_x	0.594**	−0.354	−0.117	−0.446*
pMDA	0.794**	−0.494*	−0.613**	−0.147
tMDA	–	−0.453*	−0.440*	−0.426*

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

The results shown in Table 3 revealed that plasma GSSG/GSH ratio and testicular ferric reducing antioxidant power (FRAP) showed significant increase and decrease respectively in hypothyroid group as compared to control group. On the other hand, in co-treatment group, plasma GSSG/GSH ratio and testicular FRAP showed non significant change as compared to control and folic acid groups except for plasma GSSG/GSH ratio which showed significant increase as compared to control group. In comparison to hypothyroid group, plasma GSSG/GSH ratio and testicular FRAP showed non significant change in co-treatment group. In post-treatment group, plasma GSSG/GSH ratio and testicular FRAP showed significant increase and non significant change respectively as compared to control group. In comparison to hypothyroid and co-treatment groups, plasma GSSG/GSH ratio and testicular FRAP showed non significant change in post-treatment group (Table 3).

Data evidence in Table 4 showed that while plasma testosterone and sperm count exhibited non significant change, sperm motility exhibited significant increase in folic acid group as compared to control group. On the other hand, there was significant decrease in plasma testosterone, sperm count and sperm motility in hypothyroid group as compared to control and folic acid groups. In comparison to control and folic acid groups, while plasma testosterone and sperm motility showed significant decrease, sperm count showed non significant change in co-treatment group. Moreover, in comparison to hypothyroid group, while sperm count and motility showed significant increase, plasma testosterone level showed non significant change in co-treatment group. In post-treatment group, while plasma testosterone level showed significant decrease as compared to control and folic acid groups, it showed non significant change as compared to hypothyroid and co-treatment groups. On the other hand, sperm count showed significant increase in post-treatment group as compared to other groups of the study. Sperm motility showed non significant and significant decrease as compared to control and folic acid groups respectively in post-treatment group. Meanwhile, sperm motility significantly increased in post-treatment group as compared to hypothyroid and co-treatment groups (Table 4).

3.1. Pearson correlation coefficient of different studied parameters in different studied groups

In Table 5, a significant negative correlation was detected between total T_3 and tHcy, tNO_x and plasma MDA. On the other hand, tHcy had a significant positive correlation with tNO_x and both of them had a significant positive correlation with plasma MDA in different studied groups.

Table 6 revealed that total T_3 had a significant negative correlation with testicular MDA and non significant correlation with FRAP, testosterone and sperm count. On the other hand, tHcy had a significant positive and negative correlation with testicular MDA and testosterone respectively and non significant correlation with FRAP and sperm count. The same finding was detected with tNO_x except for the non significant correlation with testosterone and the significant negative correlation with sperm count. Plasma MDA was found to have a significant positive correlation with testicular MDA and both of them had a significant negative correlation with FRAP and testosterone. However, plasma MDA and testicular MDA had a non significant correlation and significant negative correlation respectively with sperm count in different studied groups.

4. Discussion

The current study revealed that hypothyroidism did induce a loss in body weight, food intake, fluid intake and appetite. Such an observation does not agree with some previous studies, even though some studies confirm our present results [38,46] and this may be due to PTU which is associated with some common side effects as loss of appetite [8].

On the other hand, non significant change in relative testes weight was observed in different groups under study. This is not similar to other studies [38]. However, this could be explained by the different life stage in which hypothyroidism was induced. On the other hand, relative epididymides weight (REW) showed significant increase in post-treatment group as compared to other groups. This may be ascribable to the increased sperm count in this group as presented later.

The present study also revealed significant decrease and increase in T_3 and TSH levels respectively in the hypothyroid and co-treatment groups when compared to their respective controls. This finding is compatible with previous studies confirming PTU as an antithyroid drug [38].

The present study declared significant increase in plasma total homocysteine (tHcy) levels in hypothyroid group when compared

to the control and folic acid groups. This finding is in line with that of Orzechowska-Pawilojc et al. [32]. The role of hypothyroidism in elevation of tHcy was confirmed by the significant negative correlation between total T_3 and tHcy as represented in the present study.

The pathogenesis of elevated tHcy in hypothyroidism can be explained by the fact that hypothyroidism markedly affects riboflavin metabolism, mainly by reducing the activity of flavokinase and thereby the synthesis of FMN and FAD which serve as cofactors for flavoprotein methylenetetrahydrofolate reductase (MTHFR) [3,32].

It is, however, suggested, that changes in folate level [26] or in activities of methionine synthase and cystathionine-β-synthase not only MTHFR [13] may be responsible for the increased serum Hcy level in patients with hypothyroidism. An alternative explanation of this effect could be attributed to the reduced glomerular filtration rate in hypothyroidism which is linked to impaired renal Hcy clearance and hyperhomocysteinemia [44].

On the other hand, in co-treatment and post-treatment groups there was a significant decrease in Hcy level when compared to its level in hypothyroid group. This finding suggests the role of folic acid supplementation in both groups to decrease Hcy level as reported by Clarke et al. [7] and Diekman et al. [13].

Our present study showed that plasma total NO metabolites was significantly higher in the hypothyroid group when compared to the respective controls. This is consistent with the findings of Virdis et al. [46]. The significant negative correlation between total T_3 and total NO_x in the current study further confirms the relation between hypothyroidism and total NO_x elevation.

This finding may be due to increased vascular oxidative burden associated with homocysteinemia that induces NADPH oxidase and inducible nitric oxide synthase activity, contributing to increased superoxide radicals production in rat vessels [46]. Furthermore, Hcy is closely associated with endothelial dysfunction through its impact on eNOS coupling [41,36]. These superoxide radicals react with nitric oxide (NO) to form peroxynitrite radicals, leading to low endothelial NO bioavailability and endothelial dysfunction. This assumption was confirmed by the significant positive correlation between tHcy and tNO_x presented in the present study.

On the other hand, significant decrease in total NO_x in co-treatment and post-treatment groups was observed when compared to the hypothyroid group. This finding can be explained by the ability of folic acid to prevent peroxynitrite-mediated tetrahydrobiopterin oxidation and improve eNOS coupling and dimerization [31].

In hypothyroidism, a decrease in free radical production is expected because of the associated metabolic suppression [30]. However, there are some studies reporting oxidative stress in hypothyroidism [49]. The significantly higher plasma and testicular MDA levels of hypothyroid group in comparison to the respective controls reflect an enhanced oxidative stress in hypothyroidism. This relation between hypothyroidism and oxidative damage was supported by the significant negative correlation between total T_3, and plasma and testicular MDA as represented in the current study.

The enhanced oxidative stress in hypothyroidism is suggested to develop due to oxidation of membrane lipids of cells by hypothyroidism [17]. Furthermore, it is suggested to be associated with the observed hyperhomocysteinemia [10] as represented in the present study by the significant positive correlation between tHcy, and plasma and testicular MDA. Another explanation of this enhanced oxidative stress could be attributed to folate deficiency associated with hypothyroidism as reported by Diekman et al. [13].

In the co-treatment and post-treatment groups, we observed that lipid peroxidation significantly decreased in plasma and testicular homogenate as compared to hypothyroid group. This reflects the antioxidant power of folic acid against free radicals [35].

The significant increase in GSSG/GSH ratio in hypothyroid group as compared to control group is similar to that of Sahoo et al. [38]. This finding corroborates the role of thyroid hormones in triggering the biosynthesis of GSH. On the other hand, we observed significant increase in this ratio in folic acid, co-treatment and post-treatment groups in comparison to control group. This finding may be explained by the consumption of GSH in regeneration of FA–OH from (FA–O·) generated through folic acid interaction with free radicals.

Ferric reducing antioxidant power encompasses different enzymatic and non-enzymatic antioxidant factors and it is easy to be measured [14]. The significant decrease in FRAP of testicular homogenate in hypothyroid group as compared to control group reflects oxidative stress as indicated by the significant negative correlation between testicular MDA (marker of oxidative stress) and FRAP. This also reflects reduction of antioxidants effectiveness with hypothyroidism [49]. However, the resulted non significant correlation between total T_3 and FRAP may be explained by the role of folic acid in co-treatment (group IV) in restoring FRAP without restoration of euthyroid state as presented in the present study.

In addition, the non significant change in FRAP in co-treatment and post-treatment groups as compared to control and folic acid groups corroborates the antioxidant properties of folic acid and the role of thyroid hormones in antioxidants biosynthesis [45].

On the other hand, the plasma testosterone level was significantly lower in the hypothyroid group than those of the respective controls. However, the non significant correlation between total T_3 and testosterone was due to the observed effect of PTU itself in inhibiting steroidogenesis as presented later. This was also reported by Sakai et al. [39]. Nevertheless, some studies demonstrated that levels of testosterone in adult rats were unaffected by induced hypothyroidism [9]. These inconsistencies have been attributed to differences in the age, duration of treatment, and method of inducing the hypothyroid state in experimental animals [28].

Concerning the literature data, the inhibitory mechanism of hypothyroidism on testosterone production involved inhibition of mRNA expression of the steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme ($P450_{scc}$) function [6,22,33,40] Concerning the data of the present study, this decrement of plasma testosterone can be explained by the oxidative stress found herein which can directly act to reduce testosterone production in rat Leydig cells [43]. This was represented herein by the significant negative correlation between testosterone, and plasma MDA and testicular MDA.

Besides, there was non significant change in plasma testosterone level in co-treatment and post-treatment groups as compared to hypothyroid group. This finding may be ascribable to the direct action of PTU *per se* on Leydig cells to inhibit steroidogenesis [5].

Regarding spermatogenesis, impaired spermatogenesis was observed in the present study as represented by significant decrease in sperm count and motility in hypothyroid group in comparison to respective controls as also reported by Sahoo et al. [38]. However, the non significant correlation between total T_3 and sperm count in the present study may be due to the role of folic acid in co-treatment (group IV) in enhancing sperm count without restoring T_3 concentration as presented later.

This finding in the present study could be the result of several implications. First, thyroid hormone itself has been shown to play an important role in testicular physiology [42]. Second, reduced plasma testosterone level as presented herein may affect due to the role of testosterone in spermatogenesis [12]. Furthermore, oxidative stress presented herein alters the motility and the genetic integrity of sperm cells [19]. This effect was confirmed by the significant negative correlation between testicular MDA (marker of oxidative stress) and sperm count as represented by the present study.

Third, the NO signaling pathways are involved in spermatogenesis and sperm motility [25]. In this context, any alteration of NO bioavailability, e.g. by hyperhomocysteinemia, may have direct consequences on male reproductive functions. This effect was confirmed by the significant negative correlation between tNO_x and sperm count. Finally, it has also been suggested that the adverse reproductive outcome in hyperhomocysteinemia may be related to homocysteine-induced precocious atherosclerotic vascular alterations [37].

On the other hand, there was a significant increase in sperm count and motility in co-treatment and post-treatment groups as compared to hypothyroid group in the present study. This corroborates the role of folic acid in enhancing spermatogenesis [15]. Besides, the current study revealed a significant increase in sperm count in post-treatment group as compared to other groups as also reported by Joyce et al. [21].

5. Conclusions

This study indicates that post-pubertal hypothyroidism in male rats was associated with hyperhomocysteinemia, oxidative stress and other biochemical alterations. These factors may, at least in part, contribute toward testicular dysfunction, which eventually leads to the testicular degenerative biochemistry and morphology (data not shown) observed in the present study. Indeed, this postulates an indirect negative impact of post-pubertal hypothyroidism on testicular function through development of these factors.

This is plus the observed role of folic acid supplementation in enhancing spermatogenesis and building up the antioxidant status against the oxidants in the present study. Moreover, folic acid supplemented after restoration of euthyroid state as in post-treatment group (V) revealed better results than what observed when folic acid was supplemented with hypothyroidism concurrently as in co-treatment group (IV).

Consequently, folic acid supplementation enhancement of spermatogenesis will be of major interest to be used as an adjuvant therapy under these conditions. In addition, PTU itself was found to inhibit steroidogenesis, so it is not recommended to treat hyperthyroid conditions like Graves' disease.

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