1. **Folic acid alleviates oxidative stress and hyperhomocysteinemia involved in**

2 **testicular dysfunction of hypothyroid rats**

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1. **Abstract**
2. Although there is general agreement that thyroid hormone is an important hormonal
3. regulator of testis physiology during development period, its role in the post-pubertal and
4. adult testes is still controversial. Furthermore, most experimental studies to date have
5. focused on thyroid hormone effects on the developing testes and only limited data are
6. available on its role in spermatogenesis. This study evaluated some biochemical

30 alterations in post-pubertal hypothyroidism and its impact on testicular function.

1. Additionally, the ameliorating role of folic acid supplementation was investigated. Fifty
2. male albino rats were randomly divided into five groups (group I, control; group II, folic
3. acid; group III, 0.05% propylthiouracil-induced hypothyroid rats; group IV, co-treatment;

34 group V, post-treatment). Plasma total homocysteine, total NO metabolites,

1. malondialdehyde and GSSG/GSH ratio quantified by HPLC significantly (*P*<0.05)
2. increased in hypothyroid rats as compared to controls. These biochemical alterations at

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| 37 | least in part disrupted spermatogenesis in these experimental models. Folic acid |
| 38 | supplemented after restoration of the euthyroid state (group V) presented better |

1. amelioration to spermatogenesis over its concurrent supplementation (group IV). This
2. postulates an indirect negative impact of post-pubertal hypothyroidism on testicular
3. function through development of these alterations. This is plus the observed role of folic
4. acid supplementation in enhancing spermatogenesis, boosting sperm concentration and
5. building up the antioxidant status against the oxidants in the present study. If confirmed
6. in human beings, our results could propose that folic acid can be used as an adjuvant
7. therapy in hypothyroidism disorders with thyroxin replacement therapy.

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1. Keywords: Hypothyroidism; Homocysteine; Folic acid; Testes; Rat

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1. **1- Introduction**
2. In mammals, altered thyroid status is known to adversely affect many organs and tissues.
3. Nevertheless, for many years, the impact of thyroid disorders on male reproduction
4. remained controversial. Early studies in the 1950’s demonstrated that testes were
5. essentially independent of thyroid hormone effects. However, in the past two decades,
6. clinical studies have demonstrated that thyroid hormone plays an important role in
7. testicular development and function.(47)
8. It is now established that T3 regulates the maturation and growth of testis, controlling
9. Sertoli cell and Leydig cell proliferation and differentiation during testicular development
10. in rats and other mammal species.(42) These data, in conjunction to the findings that
11. thyroid hormone receptors and iodothyronine deiodinases are present in human and rat
12. testes from neonatal to adult life(2, 42), confirm that thyroid hormone plays a key role in
13. testicular development.
14. On the other hand, although there is general agreement that thyroid hormone is an
15. important hormonal regulator of testis physiology during development period, its role in
16. the post-pubertal and adult testes is still controversial. Furthermore, most experimental
17. studies to date have focused on thyroid hormone effects on the developing testes and only
18. limited data are available on its role in spermatogenesis. (47)
19. Propylthiouracil (PTU) is known to inhibit thyroid hormone synthesis and conversion of
20. peripheral T4 to T3 and thereby reduces serum T3 concentration. PTU is also used in
21. treating hyperthyroid conditions like Graves’ disease. It has been linked with certain side
22. effects such as transient leukopenia, jaundice, hepatomegaly and vasculitis.(5) Thus,

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1. chemical induction of hypothyroid state by antithyroid drugs as PTU has been widely
2. established to investigate the role of thyroid hormones in testicular physiology.(38)
3. Hypothyroidism has been reported to induce mild hyperhomocysteinemia and endothelial
4. dysfunction through reduced endothelial NO bioavailability.(46) However, the impact of
5. hyperhomocysteinemia and endothelial dysfunction on testicular function is unclear.

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| 80 | Besides, regulatory role of thyroid hormone in testicular physiology is well |  |
|  | established |  | ; however, its effect on testicular antioxidant defense system is |  |
| 81 | (38) |  |

1. inadequate.(47)
2. Folic acid has been reported to have an antioxidant power against ROS and an alleviating
3. role in hyperhomocysteinemia and the associated endothelial dysfunction.(31) Also,
4. progressive folate deficiency was suggested to develop with hypothyroidism.(13) This
5. deficiency may be responsible for reduced sperm concentration.(48) Supporting this
6. assumption, a high affinity folate binding protein has been identified in human semen and
7. prostate gland.(18) This finding supports the connection between folate status and male
8. reproductive function. This further illustrates the need for an intact folate cycle to

90 maintain normal spermatogenesis and the positive effect of folic acid on sperm

1. parameters.(15) It is, however, suggested, that changes in folate level may be responsible
2. for the increased serum Hcy level in patients with hypothyroidism. (26)
3. The present study represented a contribution to declare the effect of low thyroid hormone
4. status on total plasma homocysteine level and oxidative stress parameters. Additionally,
5. the impact of these biomarkers on testicular function in PTU-induced hypothyroidism at
6. the post-pubertal stage of male rats was investigated. It also aimed to elucidate the role of
7. folic acid supplementation in enhancing spermatogenesis, boosting sperm concentration

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1. and building up the antioxidant status as a concurrent treatment with hypothyroidism and
2. as a post-treatment after restoration of the euthyroid state.

100 **2-** **Materials and methods**

1. The experiment was performed on fifty male albino rats (*Rattus norvigicus*) weighing
2. 120 g (±10) and of 6-7 week’s age. They were obtained from our laboratory farms,
3. Zoology Department, Faculty of Science, Tanta University, Egypt. The rats were kept in
4. the laboratory for one week before the experimental work and maintained on a standard
5. rodent diet (20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5%
6. vitaminzed starch; Egyptian Company of Oils and Soap, Kafr-Elzayat, Egypt) and water
7. available *ad libitum*. The temperature in the animal room was maintained at 23±2°C with
8. a relative humidity of 55±5%. Light was on a 12:12 h light-dark cycle. All the
9. experiments were done in compliance with the guiding principles in the care and use of
10. laboratory animals. The rats were equally divided into five groups (10 animals each).
11. **Group I:** Control group in which animals never received any treatment (euthyroid).
12. **Group II:** Folic acid group in which animals received folic acid (El Nasr Pharmaceutical
13. Chemicals Co.; 0.011 µmol/g body weight/day) only for four weeks (from 2nd week to 6th
14. week after the experiment start) orally by a stomach tube. (27)
15. **Group III:** Hypothyroid group in which a chemical experimental rat model of
16. hypothyroidism that mimics hypothyroidism in humans has been developed. Rats
17. received 0.05% 6-*n*-propyl -2-thiouracil (PTU; Thyrocil®) in drinking water for 6 weeks
18. (38) to cover a complete spermatogenic cycle in rats.(11)
19. **Group IV:** Co-treatment group in which animals received 0.05% PTU in drinking water
20. and folic acid (0.011 µmol/g body weight/day) concurrently according to Matte *et al*.(27)

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1. The dose period of PTU was six weeks as in hypothyroid rats group. However, folic acid
2. was administered orally by a stomach tube for 4 weeks form the second to sixth week
3. after evidence of hypothyroidism had been established at the end of the second week after
4. the experiment start.
5. **Group V:** Post-treatment group in which animals received 0.05% PTU in drinking water
6. for 6 weeks as in hypothyroid group. Additionally, folic acid was administered for
7. another 4 weeks (from 7th week to 10th week after the experiment start) while PTU was
8. withdrawn after the sixth week to establish the euthyroid state.(38)
9. At the end of the experimental period, rats from each group were euthanized with
10. intravenous injection with sodium pentobarbital and subjected to a complete necropsy
11. after 10–12 h of fasting. Testes and epididymides were removed, carefully cleaned from
12. adhering connective tissue in cold saline, weighed and quickly stored at -20°C until
13. analysis.
14. Blood samples were individually collected from each rat and divided into two parts. The
15. first part was collected in non heparinized glass tubes to estimate serum parameters.
16. Serum was separated by centrifugation at 3000 rpm for 15 minutes. The collected serum
17. was stored at -18 °C until analysis. The second part was transferred to EDTA-containing
18. glass tubes to obtain blood plasma. Plasma samples were subjected directly to High

139 performance Liquid Chromatography (HPLC) analysis. Plasma samples were

1. deproteinized by 75% aqueous HPLC grade methanol in a ratio 1:4 (plasma : methanol)
2. v/v then centrifuged at 3000 r.p.m for 5 min at 4C and the supernatants were separated
3. and used for HPLC application.

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1. Serum was analyzed to determine the T3 and TSH levels. Determination of serum total
2. triiodothyronine (T3)(50) and thyroid stimulating hormone (TSH)(51) was carried out by
3. solid phase enzyme-linked immunosorbent assay using Biocheck kit, Inc (USA). The
4. HPLC instrument was Agilent 1200 series HPLC system from Agilent Technologies
5. (USA). HCy, GSH and GSSG were determined by HPLC using the method of Jayatilleke
6. and Shaw.(20) Nitrites and nitrates were determined according to the method of
7. Papadoyannis *et al.* (34) by HPLC. Total plasma malondialdehyde (MDA) was determined
8. by HPLC according to the method of Karatas *et al.* (23) and Karatepe (24). Testosterone
9. concentration was estimated by HPLC as described by Gonzalo–Lumbreras *et al.* (16).
10. Testicular homogenate (10%; w/v) was prepared in ice-cold 0.067M phosphate buffer
11. (pH=7) then, the homogenate was centrifuged at 3000 r.p.m for 10 min. at 4°C. The
12. resulting supernatant was used to determine the testicular total antioxidat capacity (TAC)
13. and MDA content. TAC or ferric reducing antioxidant power (FRAP) was determined
14. according to Benzie and Strain.(1) The method measures the ferric reducing ability of
15. testicular homogenate . Thiobarbituric acid reactive substance (TBARS) level or
16. malondialdehyde (MDA) in the testicular homogenate was estimated by the method of
17. Mesbah *et al*. (29).
18. By laparotomy, the left and right caudal parts of the epididymis were carefully separated
19. from the testes, finely minced in 5 ml of Hanks’ buffered salt medium, and incubated at
20. room temperature for 15 min to provide the migration of all spermatozoa from
21. epididymal tissue to fluid.(4) The diluted sperm suspension (10 ml) was transferred to the
22. hemocytometer (Improved Neubauer, Weber, UK), and the settled sperm were counted
23. with a light microscope at 400× magnification (million/ml). Then, the sperm count was

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1. calculated relative to the epididymal wt. (Sperm/g). The motility assay was conducted by
2. observing the sperm suspension on a slide glass at 37˚C. The percentage of motile
3. spermatozoa was determined by counting more than 200 spermatozoa randomly in 10
4. selected fields under a light microscope (Olympus microscope), and the mean number of
5. motile sperm × “100/total number of sperms” was calculated. (4)
6. Results were analyzed using one-way analysis of variance (ANOVA) followed by the
7. Least Significant Difference (LSD) tests to compare between different groups. Data were
8. presented as the mean±SEM. *P* values less than 0.05 were considered significant. Pearson
9. correlation coefficient (r): the reliability of an estimate depends on the relationship
10. between two variables and measure of this closeness is such a measure, commonly
11. symbolized as "r". All statistical analyses were performed using SPSS statistical version
12. 16 software package (SPSS® Inc., USA).

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| 179 | **3- Results** |

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

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1. Serum triiodothyronine (T3) and thyroid stimulating hormone (TSH) levels showed
2. significant (*p*<0.05) decrease and increase respectively in hypothyroid and co-treatment
3. groups (group III and IV) as compared to control and folic acid groups (group I and II).
4. Meanwhile, there was non significant change in T3 and TSH levels in folic acid and post-
5. treatment groups (group II and V) as compared to control group (**Table 2**).
6. Plasma levels of total homocysteine (tHcy) and total NO metabolites (NOx) showed
7. significant increase in hypothyroid group as compared to control and folic acid groups
8. (group I, II). In comparison to hypothyroid group, plasma levels of tHcy and total NOx
9. showed significant decrease in co-treatment group. Besides, in post-treatment group,
10. while plasma levels of total NOx showed non significant change, plasma level of tHcy
11. showed significant increase as compared to control and folic acid groups. In comparison
12. to hypothyroid group, plasma levels of tHcy and total NOx showed significant decrease in
13. post-treatment group. In comparison to co-treatment group, while plasma levels of total
14. NOx showed significant decrease, plasma levels of tHcy showed non significant change
15. in post-treatment group (**Table 2**).
16. In **Table 3**, there was significant increase in plasma and testicular MDA levels in
17. hypothyroid group as compared to control and folic acid groups. On the other hand,
18. plasma and testicular MDA levels showed significant and non significant increases
19. respectively in co-treatment group as compared to control and folic acid groups. In
20. comparison to hypothyroid group, plasma and testicular MDA levels showed significant
21. decrease in co-treatment group. Moreover, plasma and testicular MDA levels showed
22. significant increase in post-treatment group as compared to control and folic acid groups
23. except for testicular MDA level which showed non significant change as compared to

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1. folic acid group. Meanwhile, plasma and testicular MDA levels showed significant
2. decrease in post-treatment group as compared to hypothyroid group. In comparison to co-
3. treatment group, plasma and testicular MDA levels showed significant and non
4. significant increase respectively in post-treatment group.
5. The results shown in **Table 3** revealed that plasma GSSG/GSH ratio and testicular Ferric

216 reducing antioxidant power (FRAP) showed significant increase and decrease

1. respectively in hypothyroid group as compared to control group. On the other hand, in
2. co-treatment group, plasma GSSG/GSH ratio and testicular FRAP showed non
3. significant change as compared to control and folic acid groups except for plasma
4. GSSG/GSH ratio which showed significant increase as compared to control group. In
5. comparison to hypothyroid group, plasma GSSG/GSH ratio and testicular FRAP showed
6. non significant change in co-treatment group. In post-treatment group, plasma
7. GSSG/GSH ratio and testicular FRAP showed significant increase and non significant
8. change respectively as compared to control group. In comparison to hypothyroid and co-
9. treatment groups, plasma GSSG/GSH ratio and testicular FRAP showed non significant
10. change in post-treatment group (**Table 3**).
11. Data evidence in **Table 4** showed that while plasma testosterone and sperm count
12. exhibited non significant change, sperm motility exhibited significant increase in folic
13. acid group as compared to control group. On the other hand, there was significant
14. decrease in plasma testosterone, sperm count and sperm motility in hypothyroid group as
15. compared to control and folic acid groups. In comparison to control and folic acid groups,
16. while plasma testosterone and sperm motility showed significant decrease, sperm count
17. showed non significant change in co-treatment group. Moreover, in comparison to

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1. hypothyroid group, while sperm count and motility showed significant increase, plasma
2. testosterone level showed non significant change in co-treatment group. In post-treatment
3. group, while plasma testosterone level showed significant decrease as compared to
4. control and folic acid groups, it showed non significant change as compared to
5. hypothyroid and co-treatment groups. On the other hand, sperm count showed significant
6. increase in post-treatment group as compared to other groups of the study. Sperm motility
7. showed non significant and significant decrease as compared to control and folic acid
8. groups respectively in post-treatment group. Meanwhile, sperm motility significantly
9. increased in post-treatment group as compared to hypothyroid and co-treatment groups
10. (**Table 4**).
11. **Pearson correlation coefficient of different studied parameters in different studied groups**
12. In **Table 5**, a significant negative correlation was detected between total T3 and tHcy,
13. tNOx and plasma MDA. On the other hand, tHcy had a significant positive correlation
14. with tNOx and both of them had a significant positive correlation with plasma MDA in
15. different studied groups.
16. **Table 6** revealed that total T3had a significant negative correlation with testicular MDA
17. and non significant correlation with FRAP, testosterone and sperm count. On the other
18. hand, tHcy had a significant positive and negative correlation with testicular MDA and
19. testosterone respectively and non significant correlation with FRAP and sperm count.
20. The same finding was detected with tNOx except for the non significant correlation with
21. testosterone and the significant negative correlation with sperm count. Plasma MDA was
22. found to have a significant positive correlation with testicular MDA and both of them had
23. a significant negative correlation with FRAP and testosterone. However, plasma MDA

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1. and testicular MDA had a non significant correlation and significant negative correlation
2. respectively with sperm count in different studied groups.

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260 **4-** **Discussion**

1. Thyroid hormones disturbances are associated with different degrees of thyroid failure
2. and metabolic consequences.(42) In contrast to most studies in this field, the hypothyroid
3. condition was induced in the current study during puberty and not immediately after
4. birth. This by itself is novel as it tackles another part of the testicular differentiation
5. process.
6. The current study revealed that hypothyroidism did induce a loss in body weight, food
7. intake, fluid intake and appetite. Such an observation does not agree with some previous
8. studies where body gain has been reported to occur, even though some studies confirm
9. our present results. (38,46) This is may be due to the induction of hypothyroidism by PTU
10. which is associated with some common side effects as loss of appetite. (8)
11. On the other hand, non significant change in relative testes weight (RTW) was observed
12. in different groups under study. This is not similar to other studies. (38) However, this
13. could be explained by the different life stage in which hypothyroidism was induced. On
14. the other hand, relative epididymides weight (REW) showed significant increase in post-
15. treatment group as compared to other groups. This may be ascribable to the increased
16. sperm count in this group as presented later.
17. The present study also revealed significant decrease and increase in T3 and TSH levels
18. respectively in the hypothyroid and co-treatment groups when compared to their
19. respective controls. This might be considered as a sound argument in the induction of

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1. hypothyroidism indicating that PTU is a good choice as an antithyroid drug for induction
2. of hypothyroid state. This finding is compatible with previous studies. (38)
3. The present study contributed information towards establishing an association between
4. hypothyroidism and hyperhomocysteinemia as it declared significant increase in plasma
5. total homocysteine (tHcy) levels in hypothyroid group when compared to the control and
6. folic acid groups. This finding is in line with that of Orzechowska-Pawilojc *et al*.(32). The
7. role of hypothyroidism in elevation of tHcy was confirmed by the significant negative
8. correlation between total T3 and tHcy as represented in the present study.
9. The pathogenesis of elevated tHcy in hypothyroidism can be explained by the fact that
10. thyroid hormones markedly affect riboflavin metabolism, mainly by stimulating
11. flavokinase and thereby the synthesis of flavin mononucleotide (FMN) and flavin
12. adeninedinucleotide (FAD) which serve as cofactors for homocysteine/methionine cycle
13. enzymes. (3) Hypothyroid animals can be defective in converting riboflavin to the co-
14. enzyme FAD, and consequently, deficient in the flavoprotein methylenetetrahydrofolate
15. reductase (MTHFR) activity. (32)
16. It is, however, suggested, that changes in folate level (26) or in activities of methionine
17. synthase and cystathionine-β-synthase not only MTHFR(13) may be responsible for the
18. increased serum Hcy level in patients with hypothyroidism. An alternative explanation of
19. this effect could be attributed to the reduced glomerular filtration rate in hypothyroidism
20. which is linked to impaired renal Hcy clearance and hyperhomocysteinemia. (44)
21. On the other hand, in co-treatment and post-treatment groups there was a significant
22. decrease in Hcy level when compared to its level in hypothyroid group. This finding
23. suggests the role of folic acid supplementation in both groups to decrease Hcy level as

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1. reported by Clarke *et al*. (7). Noteworthy, in post-treatment group, restoration of euthyroid
2. state shared with folic acid supplementation in lowering the level of Hcy. This is in
3. agreement with Diekman *et al*. (13).
4. Our present study showed that plasma total NO metabolites was significantly higher in
5. the hypothyroid group when compared to the respective controls. This is consistent with
6. the findings of Virdis *et al*.(46). The significant negative correlation between total T3 and
7. total NOx in the current study further confirms the relation between hypothyroidism and
8. total NOx elevation.
9. This finding may be due to increased vascular oxidative burden associated with
10. homocysteinemia that induces NADPH oxidase and inducible nitric oxide synthase
11. activity, contributing to increased superoxide radicals production in rat vessels.(46)
12. Furthermore, Hcy is closely associated with endothelial dysfunction through its impact on
13. eNOS coupling. (41) A decreased supply of eNOS substrate L-arginine and diminished

316 tetrahydrobiopterin bioavailability observed in homocysteinemia, have been

1. demonstrated to induce eNOS uncoupling and superoxide radicals production in cell
2. cultures of endothelial cells.(36) These superoxide radicals react with nitric oxide (NO) to
3. form peroxynitrite radicals, leading to low endothelial NO bioavailability and endothelial
4. dysfunction. This assumption was confirmed by the significant positive correlation
5. between tHcy and tNOx presented in the present study.
6. On the other hand, significant decrease in total NOx in co-treatment and post-treatment
7. groups was observed when compared to the hypothyroid group. This finding can be
8. explained by the ability of 5-MTHF, the circulating form of folic acid to prevent
9. peroxynitrite-mediated tetrahydrobiopterin oxidation and improve eNOS coupling and

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326 dimerization. It is due to its ability to increase endothelial tetrahydrobiopterin

1. bioavailability in vessels through scavenging free radicals responsible for its oxidation.
2. (31) This was compatible with the significant positive correlation between plasma MDA
3. (marker of oxidative stress) and tNOx.
4. On the other hand, the decrease in total NOx in post-treatment group was significant in
5. comparison to co-treatment group. This is similar to the study of Virdis *et al*.(46).
6. In hypothyroidism, a decrease in free radical production is expected because of the
7. associated metabolic suppression.(30) However, there are some studies reporting oxidative
8. stress in hypothyroidism. (49)
9. The significantly higher plasma and testicular MDA levels of hypothyroid group in

336 comparison to the respective controls reflect an enhanced oxidative stress in

1. hypothyroidism. This is similar to the results of Sahoo *et al*.(38). This relation between
2. hypothyroidism and oxidative damage was supported by the significant negative
3. correlation between total T3, and plasma and testicular MDA as represented in the current
4. study.
5. The enhanced oxidative stress in hypothyroidism is suggested to develop due to oxidation
6. of membrane lipids of cells by hypothyroidism.(17)Furthermore, it is suggested to be
7. associated with the observed hyperhomocysteinemia as represented in the present study
8. by the significant positive correlation between tHcy, and plasma and testicular MDA.
9. Hcy is readily oxidized as a consequence of auto-oxidation leading to the formation of
10. homocystine, homocysteine-mixed disulfides, and homocysteine thiolactone. During
11. oxidation of the sulfhydryl group, free radicals are generated, which account for the
12. endothelial cytotoxicity of homocysteine. (10)

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1. This contributes to increased superoxide radicals production in rat vessels.(36) These free
2. radicals can initiate lipid peroxidation as marked by increased level of MDA. Another
3. explanation of this enhanced oxidative stress could be attributed to folate deficiency
4. associated with hypothyroidism as reported by Diekman *et al*. (13). Folate deficiency

353 reduces Phosphatidylethanolamine methylation and could thus alter membrane

1. phospholipids organization and function. (10)
2. In the co-treatment and post-treatment groups, we observed that lipid peroxidation
3. significantly decreased in plasma and testicular homogenate as compared to hypothyroid
4. group. This reflects the antioxidant power of folic acid against free radicals. (35)
5. Also, the present study demonstrated the role of folic acid in reducing Hcy accumulation
6. responsible in part for oxidative damage. It is of importance to note that restoration of
7. euthyroid state in post-treatment group shared with folic acid in lowering lipid
8. peroxidation. This is through its regulation of oxidative metabolism, protein and
9. antioxidant enzymes synthesis and degradation.(45) On the other hand, the observed
10. significant increase in plasma and testicular MDA levels in post-treatment group as
11. compared to control group is in agreement with Sahoo *et al*.(38)
12. The significant increase in GSSG/GSH ratio in hypothyroid group as compared to control
13. group is similar to that of Sahoo *et al*.(38). This finding corroborates the role of thyroid
14. hormones in triggering the biosynthesis of GSH and the role of GSH *per se* in scavenging
15. free radicals leading to depletion of GSH. On the other hand, we observed significant
16. increase in this ratio in folic acid, co-treatment and post-treatment groups in comparison
17. to control group. This finding may be explained by the consumption of GSH in

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1. regeneration of FA-OH from (FA-O) generated through folic acid interaction with free
2. radicals.
3. FRAP encompasses different enzymatic and non-enzymatic antioxidant factors and it is
4. easy to be measured. (14) Studies of thyroid hormone effect on testicular antioxidant
5. defense system are inadequate. (38) Nevertheless, the significant decrease in FRAP of
6. testicular homogenate in hypothyroid group compared to control group reflects oxidative
7. stress as indicated by the significant negative correlation between testicular MDA
8. (marker of oxidative stress) and FRAP. This also reflects reduction of antioxidants
9. effectiveness with hypothyroidism as also reported by Yilmaz *et al*.(49). However, the
10. resulted non significant correlation between total T3 and FRAP may be explained by the
11. role of folic acid in co-treatment (group IV) in restoring FRAP without restoration of
12. euthyroid state as presented in the present study.
13. In addition, the non significant change in FRAP in co-treatment and post-treatment
14. groups as compared to control and folic acid groups corroborates the antioxidant
15. properties of folic acid and the role of thyroid hormones in antioxidants biosynthesis.(45)
16. Although the effect of Hcy on male reproductive system is unknown, it was reported that
17. there may be a positive correlation between the increase in plasma Hcy level and
18. reduction of semen parameters.(48)
19. In the present study, the plasma testosterone level was significantly lower in the
20. hypothyroid group than those of the respective controls. However, the non significant
21. correlation between total T3 and testosterone was due to the observed effect of PTU itself
22. in inhibiting testosterone production as presented later. This was also reported by Sakai *et*
23. *al*.(39). Nevertheless, some studies demonstrated that levels of testosterone in adult rats

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1. were unaffected by induced hypothyroidism.(9) These inconsistencies have been attributed
2. to differences in the age, duration of treatment, and method of inducing the hypothyroid
3. state in experimental animals.(28)
4. Concerning the literature data, the inhibitory mechanism of hypothyroidism on
5. testosterone production involved inhibition of mRNA expression of the steroidogenic
6. acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme
7. (P450scc) function.(6, 22)
8. Concerning the data of the present study, this decrement of plasma testosterone can be
9. explained by the direct inhibitory effect of hyperhomocysteinemia as indicated by the
10. significant negative correlation between tHcy and testosterone and as reported by
11. Papadopoulos *et al*. (33). On the other hand, the oxidative stress found herein can directly
12. act to reduce testosterone production in rat Leydig cells.(43) This was represented herein
13. by the significant negative correlation between testosterone, and plasma MDA and
14. testicular MDA.
15. Besides, there was non significant change in plasma testosterone level in co-treatment
16. and post-treatment groups as compared to hypothyroid group. This finding may be
17. ascribable to the direct action of PTU *per se* on Leydig cells to inhibit steroidogenesis.(5)
18. Regarding spermatogenesis, impaired spermatogenesis was observed in the present study
19. as represented by significant decrease in sperm count and motility in hypothyroid group
20. in comparison to respective controls as also reported by Sahoo *et al*. (38). However, the
21. non significant correlation between total T3 and sperm count in the present study may be
22. due to the role of folic acid in co-treatment (group IV) and post-treatment (group V) in
23. enhancing sperm count as presented later.

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1. This finding in the present study could be the result of several implications. First, thyroid
2. hormone itself has been shown to play an important role in testicular physiology.(42)
3. Moreover, reduced plasma testosterone level as presented herein may affect due to the
4. role of testosterone in spermatogenesis.(12) Second, thiol metabolism is important for the
5. stabilization of sperm membranes and the protection of sperm DNA against damage.(15)
6. The auto-oxidation of Hcy leads to the formation of homocystine, homocysteine
7. thiolactone and sulfydryl group. Homocysteine thiolactone is a highly reactive Hcy

424 derivative that can react easily with proteins. The increase in plasma level of

1. homocysteine thiolactone blocks intracellular protein-carboxyl methylation reaction,
2. which results in the inhibition of sperm motility.(40) Furthermore, oxidative stress
3. presented herein alters the motility and the genetic integrity of sperm cells.(19) This effect
4. was confirmed by the significant negative correlation between testicular MDA and sperm
5. count as represented by the present study.
6. Third, The NO signaling pathways are involved in spermatogenesis and sperm

431 motility.(25) In this context, any alteration of NO bioavailability, e.g. by

1. hyperhomocysteinemia, may have direct consequences on male reproductive functions.
2. This effect was confirmed by the significant negative correlation between tNOx and
3. sperm count. Fourth, progressive folate deficiency was suggested to develop with
4. hypothyroidism according to Diekman *et al*. (13). This deficiency may be responsible for
5. reduced sperm concentration.(48) Finally, it has also been suggested that the adverse
6. reproductive outcome in hyperhomocysteinemia may be related to homocysteine-induced
7. precocious atherosclerotic vascular alterations, impairing the blood flow in the testicular
8. arteries. (37)

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1. On the other hand, there was a significant increase in sperm count and motility in co-
2. treatment and post-treatment groups as compared to hypothyroid group in the present
3. study. This corroborates the role of folic acid in enhancing spermatogenesis which agrees
4. with the previously reported benefits of folic acid supplementation on sperm quality and
5. male fertility. (15) Besides, the current study revealed a significant increase in sperm count
6. in post-treatment group as compared to other groups. Concomitantly, Joyce *et al*. (21)
7. reported that transient neonatal PTU-induced hypothyroidism increased daily sperm
8. production in adult rats and mice.

448 **5-** **Conclusions**

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

455 This is plus the observed role of folic acid supplementation in enhancing

1. spermatogenesis, boosting sperm concentration and building up the antioxidant status
2. against the oxidants in the present study. Moreover, folic acid supplemented after
3. restoration of euthyroid state as in post-treatment group (V) revealed better results than
4. what observed when folic acid was supplemented with hypothyroidism concurrently as in
5. co-treatment group (IV).
6. Consequently, post-pubertal hypothyroid patients will be interested to know that they are
7. in risk of possible azoospermia. Also, folic acid supplementation enhancement of

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1. spermatogenesis will be of major interest to be used as an adjuvant therapy under these
2. conditions. In addition, PTU itself was found to inhibit steroidogenesis, so it is not
3. recommended to treat hyperthyroid conditions like Graves disease.

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467 **6- References**

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1. **Legends of tables:**

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1. **Table 1:** Food intake (g/rat/day), fluid intake (ml/rat/day), increase rate of body weight
2. per week(IRBW; %), relative testes weight (RTW; g/100g) and relative epididymides
3. weight (REW; g/100g) in different groups under study.
4. **Table 2:** Triiodothyronine (T3; ng/dl), thyroid stimulating hormone (TSH; µIU/ml), total
5. plasma homocysteine (tHcy; µmol/l) and plasma total nitric oxide metabolites (tNOx;
6. µmol/l) levels in different groups under study.
7. **Table 3:** Plasma malondialdehyde (pMDA; nmol/l), testicular malondialdehyde (tMDA;
8. nmol/g), plasma GSSG/GSH raio (pGSSG/GSH) and testicular ferric reducing
9. antioxidant power (tFRAP; µmol Fe+2/g) levels in different groups under study.
10. **Table 4:** Plasma testosterone (ng/ml), sperm count (No. /g epididymis×106) and sperm
11. motility (%) in different groups under study.
12. **Table 5**: Correlation coefficient (r) of T3, tHcy and tNOx with T3, tHcy, tNOx and
13. pMDA in different studied groups.
14. **Table 6**: Correlation coefficient (r) of T3, tHcy, tNOx, pMDA and tMDA with tMDA,
15. FRAP, testosterone and sperm count in different studied groups.

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**Tables**

**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/100g) and relative epididymides weight (REW; g/100g) in different groups under study.

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

Data are expressed as mean ± S.E.M of ten 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. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment; 6W: in the initial six weeks, 4W: in the extra four weeks).

**Table 2:** Triiodothyronine (T3; ng/dl), thyroid stimulating hormone (TSH; µIU/ml), total plasmahomocysteine (tHcy; µmol/l) and plasma total nitric oxide metabolites (tNOx; µmol/l) levels in different groups under study.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Group I** | **Group II** | **Group III** | **Group IV** | **Group V** |
|  |  |  |  |  |  |
| T3 | 155.8±13.57a | 156.6±14.86a | 56±4.93b | 40.4±0.81b | 151.2±13.56a |
|  |  |  |  |  |  |
| TSH | 0.072±0.0086a | 0.051±0.0123a | 3.780±0.3470b | 4.180±0.2354b | 0.050±0.0152a |
|  |  |  |  |  |  |
| tHcy | 1.369±0.05a | 1.277±0.04a | 2.301±0.03b | 2.041±0.08c | 1.910±0.03c |
|  |  |  |  |  |  |
| tNOx | 31.404±1.66a | 38.611±2.02a,c | 52.237±3.15b | 42.289±5.10c | 33.684±1.60a |

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. 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±0.83a | 27.291±3.25a | 309.829±10.32b | 130.943±10.16c | 218.252±8.39d |
|  |  |  |  |  |  |
| tMDA | 47.429±4.79a | 49.714±7.11a,c | 130.000±9.90b | 72.571±7.92a,c | 73.143±11.48c |
|  |  |  |  |  |  |
| pGSSG/GSH | 0.506±0.014a | 0.612±0.022b | 0.579±0.009b | 0.603±0.021b | 0.612±0.022b |
|  |  |  |  |  |  |
| tFRAP | 1.67±0.19a | 1.29±0.23a,b | 0.93±0.07b | 1.37±0.23a,b | 1.16±0.07a,b |

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. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment).

**Table 4:** Plasma testosterone (ng/ml), sperm count (No. /g epididymis×106) and sperm motility(%) in different groups under study.

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

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. Group I (control); Group II (folic acid); Group III (hypothyroid); Group IV (co-treatment); Group V (post-treatment).

**Table 5**: Correlation coefficient (r) of T3, tHcy and tNOx with T3, tHcy, tNOx and pMDA indifferent studied groups.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **T3** | **tHcy** | **tNOx** | **pMDA** |
|  |  |  |  |  |
| **T3** | ---- | -0.708\*\* | -0.663\*\* | -0.496\* |
|  |  |  |  |  |
| **tHcy** | -0.708\*\* | ---- | 0.539\*\* | 0.880\*\* |
|  |  |  |  |  |
| **tNOx** | -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 T3, tHcy, tNOx, pMDA and tMDA with tMDA, FRAP,testosterone and sperm count in different studied groups.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **tMDA** | **FRAP** | **Testosterone** | **Sperm count** |
|  |  |  |  |  |
| **T3** | -0.575\*\* | 0.287 | 0.315 | 0.339 |
|  |  |  |  |  |
| **tHcy** | 0.739\*\* | -0.280 | -0.631\*\* | -0.191 |
|  |  |  |  |  |
| **tNOx** | 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).