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Ameliorating Role of Folic Acid in Eltroxine Induced Hyperthyroid and Oxidative Stress in Rat Cortex, Hypothalamus and Hippocampus

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ABSTRACT

Normal brain development requires the presence of thyroid hormones (TH). The present study aimed to declare the effect of hyperthyroidism on oxidative stress parameters in brain tissues (cortex, hypothalamus and hippocampus) and the role of folic acid supplementation in treatment. Thirty adult rats were divided into 5 groups (Group 1, Control; Group 2, Folic acid; Group 3, Hyperthyroid; Group 4, Co-treated hyperthyroid with folic acid; Group 5, Post treated hyperthyroid with folic acid). The current results showed a significant increase in the concentrations of T3 & T4 in hyperthyroid rats when compared with control group. In contrast, TSH showed a significant decrease in hyperthyroid rats when compared with the control group. Calcium was significantly increased in hyperthyroid group when compared with control group. Catalase and MDA were increased, while total protein in brain tissues was decreased in hyperthyroid rats (G3) when compared with control group. Total thiol in brain tissues showed a significant decrease in hyperthyroid group when compared with control group. Treatment with folic acid showed enhancement in oxidative stress parameters in post treated group more than co treated group.

1. INTRODUCTION

Thyroid hormones (TH) have important physiological functions, not only during brain maturation but also in the adult vertebrate brain (Ahmed et al., 2010). Normal brain development requires the presence of thyroid hormones that are essential for cell migration, dendrite and axon outgrowth, synapse formation, myelination and gliogenesis (Oppenheimer and Schwartz, 1997; Ortiga-Carvalho et al., 2016). It is well-known that a higher T3 level, a hyper metabolic state, causes calorigenesis in two ways. The first is a short-term signaling mechanism with the allosteric activation of cytochrome-C oxidase and the second is a long-term pathway producing nuclear and mitochondrial gene transcription through T3 signaling, thus stimulating basal thermo genesis (Oppenheimer et al., 1994). This last mechanism causes the synthesis of the enzymes involved in energy metabolism and the components of the respiratory-chain apparatus, leading to a higher capacity of oxidative phosphorylation (Soboll, 1993; Videla, 2000). These short- and long-term pathways are mainly responsible for the increased cellular respiration caused by the hyperthyroid state. Many side effects induced by hyperthyroidism as weight loss and depression (Duyff et al., 2000 ; Silva and Bianco, 2008). Folic acid is water-soluble vitamins, which are essential in our life. Numerous clinical trials using folic acid for prevention of cardiovascular disease, stroke, cognitive decline, and neural tube defects have been completed or are underway (Massoud et al., 2012). Folic acid plays an important role in hypothyroidism in brain tissues (Tousson et al., 2012). Because TH modulates many functions, if TH levels change, many cellular processes could be altered, including modifications in the REDOX environment. Therefore the aim of the present study was to evaluate the role of folic acid on hyperthyroid induced oxidative stress in rats.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Thyroxin was purchased from Glaxosmithkline company, Folic acid was purchased from El Nasr Pharmaceutical Chemicals. Rat T3, T4, TSH Elisa chemicals from Diagnostic systems Laboratories (Taxes, USA).Serum calcium was assayed by using commercial test supplied by SPECTRUM kit. Fine chemicals obtained from Sigma Chemical Co., U.S.A. and all other chemicals and reagents were of high analytical grade.

2.1.2 Animals

The experiment was performed on 30 male albino rats weighing 120±10g and of 8-9 week's age. They were obtained from Helwan laboratory farms for the Egyptian Organization for Vaccine and Biologic Preparations. The rats were kept in the laboratory under constant condition of temperature in the animal house of Zoology Department Faculty of Science, Tanta University and maintained at 23±2^{O}C with a relative humidity of 55±5% and light was on a 12:12-h light-dark cycle for two weeks before and throughout the experimental work. The experimental protocol was approved by Local Ethics Committee and Animals Research 77 2.3.

2.1.3 Experimental groups:

Thirty rats were equally divided into five groups (6 rats each) that received the treatment via gastric intubation.

G_1: control group that included animals that received vehicle (saline).

G_2: Folic acid group in which animals daily received folic acid at a dose of (8 mg/kg BW/day) for four weeks (Matte et al., 2007).

G_3: Hyperthyroid group in which rats received L-Thyroxin sodium administration (100 µg/Kg BW/day for four weeks). (Caminos et al. 2002).

G_4: Co-treated hyperthyroid with folic acid, (Rats received both folic acid and L-Thyroxin as groups G2and G3 at the same time)

G_5: Post treated hyperthyroid rats with folic acid group, (Rats received L-Thyroxin as group G3 for 4 weeks then L-Thyroxin stopped and rats received folic acid as G2 for another 4 weeks)

2.2. Methods:

Blood collection and brain Dissection

2.2.1 Blood collection.

At the end of the experimental period, rats were euthanized through intravenous injection with sodium pentobarbital and subjected to a complete necropsy. Blood samples were individually obtained from the inferior vena cava of each rat in a glass tubes. Serum was separated by centrifugation at 3000 rpm for 15 minutes. The collected serum was stored at -80 °C until analysis.

2.2.2 Brain Dissection.

Surgical scissors, Narrow pattern forceps, Iris scissors, Metal iron-free plate, Razor blade, Scalpel handle and large forceps, were used:

a) Brain Removal

1. Cervical dislocation was used to prevent pre- and postsynaptic effects of anesthesia and a surgical scissor to remove the head with a cut posterior from the ears. Using the scissors, we made amidline incision in the skin. The skin Flipped over the eyes to free the skull, Making a small incision (Iris scissors) on the top of the skull starting from the caudal part at the point of the (inter) parietal bone, Making a firm cut through the most anterior part of the skull, between the eyes .This enables to remove the brain more easily.
2. Tilted one side of the parietal bone with the curved narrow pattern forceps and break it off. The same was done with the other side. Most likely the frontal bone will remain. In that case, a small incision was made that enabled tilting and breaking off this bone plate. We were careful of the meninges that are surrounding the brain and that are between the brain and the skull; they could rupture the brain while breaking off the skull.
3. When the brain is freed from meninges, slide the curved narrow pattern forceps (closed) under anterior part of the brain (olfactory bulb) and tilt the brain gently upward. Slide the forceps further down to break the optic nerves and other cranial nerves and gently the brain lift out of the skull.
4. The brain transferred to metal plate placed on ice to cool down the brain immediately. Excess blood was wiped off.

b) Cerebellum dissection

The brain Placed with the dorsal side facing the metal plate, lifting with the curved narrow pattern forceps the medulla/ pons upward. Using the small curved forceps (Graefe Forceps – 0.5 mm tip), cut through the pons by closing the forceps around the tissue. When the majority of the white tissue was removed, the brain turned around with the ventral side facing the metal plate. the small forceps was Placed between the cortical lobes and the cerebellum, and the cerebellum snappd off from the colliculus inferior. 6. Finally, possible remaining parts of the pons were removed.

c) Tissues dissection

The brain was placed with the ventral side facing the metal plate, the small curved forceps Placed between the cerebral halves in a closed position. Gently hold the brain in position with the large curved forceps.

Gently open the forceps, thereby slowing the opening of the cortical halves.

Repeat this process of placing the closed forceps in between the cortical halves, and opening the forceps. The initial white-colored part encountered is most likely the corpus callosum, under it is the hippocampus. Once an opening was obtained for 60% along the midline, we directed the forceps (closed position) 30–40° counterclockwise to open up the left cortex from the hippocampus by repeatedly opening the forceps. Thereafter repeating the same for the right cortex by pointing the forceps in a 30–40 clockwise direction. Repeating this movement on either side until the upper part of the hippocampus was visible. Using the large forceps, we gently pick up the cortex. By turnning the small forceps to free the hippocampus from the cortex without damaging the cortex. Again repeating the process of opening and closing the small forceps while moving them to the caudal part of the hippocampus/ cortex boundary. Once at the most caudal part of the hippocampus/cortex boundary, we moved the small forceps through the cortex. Possible remainders of cortex, visible from a more pink/yellow color than the hippocampus (gray, translucent), can be removed at that moment by snapping it off using the small forceps. Repeating the previous steps to remove the left cortex from the hippocampus. We moved the cortical halves anterior from the cortex to reveal the fornix. Using the small forceps cut the hippocampus separate from the fornix. In addition, we separate the two halves of the hippocampus. Hypothalamus located under hippocampus which found in third ventricles removed easily.

2.3 Methods

2.3.1Measurement of blood parameters:

Serum T3 and T_4 was assayed by using commercial test supplied by the Diagnostic systems Laboratories (Taxes, USA) according to the method of (Chopra et al., 1971). Serum TSH was assayed by using commercial Kit supplied by Coat-A-Count TSH IRMA (Los Angeles, USA) according to the method of (Engel,1980). Enzyme Linked Immunosorbent Assay (ELISA) test is determined by Competitive Enzyme Immunoassay. The essential reagents required for a solid phase enzyme immunoassay are including immobilized antibody, enzyme-antigen conjugate and native antigen.

Calcium was determined in serum by the colorimetric method using available commercial kits (Spectrum) according to the method described by (Barnett, 1965).

2.3.2 Measurement of brain tissues parameters:

Brain was divided into 3 tissues, cortex, hypothalamus and hippocampus and part homogenized (10% W/V) in phosphate potassium buffer (0.01 M pH , 7.4) for estimation of catalase, T. thiol and T. protein and part of the brain homogenized in KCl solution (1.15 M) for estimation of MDA, by homogenizer (Hettich model EBA 12R, Germany).

Catalase enzyme activity was measured by monitoring H_2O_2 decomposition at 240 nm according to the method described by (Xu et al., 1997). Malondialdehyde (MDA) is one of the terminal products, formed at the time of the decomposition of the polyunsaturated fatty acids mediated by free radicals. MDA was detected by TBARS analysis and measured as reported by (Mesbah et al., 2004). Total thiol was detected by using DTNB reagent as described by (Sedlak and Lindsay, 1968).Total protein concentration was detected, by the method of (Lowry et al., 1951) as modified by (Tsuyosh and James, 1978).

2.4 Statistical analysis

The data obtained in the experiment were expressed in terms of mean ± SEM. Statistical significance of data variations were assessed by one way analysis of variance (ANOVA) followed by a comparison between different groups using "Tukey-Kramer" multiple comparison t-test, which compare between all groups and showed the significant effect of treatment (Graph pad Instate software). A value of $P<0.05$ was considered to be statistically significant.

3. RESULTS

Table 1 showed a significant increase ($P\leq0.001$) in the concentration of T3 in G3when compared with G1, while, there is a significant decrease ($P\leq0.001$) in G4 and G5 when compared with G3. G5 showed a significant decrease ($P\leq0.001$) when compared with G4. Table 1 showed a significant increase ($P\leq0.001$) in T4 in G3 when compared with G1. On the other hand, there is a significant decrease ($P\leq0.001$) in G4 and G5 when compared with G3. Table 1 showed a significant decrease ($P<0.001$) in TSH in G3 when compared with G1and G2. Figure 4 showed a significant increase ($P\leq0.01$) in the concentration of calcium in G3 when compared with G1. Table 2 showed a significant increase ($P<0.001$) in catalase enzyme activity in cortex, hypothalamus and hippocampus in G3 when compared with G1; there was a significant decrease in G4 ($P<0.05$) and G5 ($P<0.001$) when compared with G3 in cortex, hypothalamus, while hippocampus showed significant decrease in G4 ($P<0.05$) and G5 ($P<0.01$) when compared with G3 . Table 3 showed a significant increase in MDA in cortex, hypothalamus

(P<0.001) and hippocampus (P<0.01) in G3 when compared with G1. G5 showed a significant decrease (P<0.05) in MDA concentration when compared with G3 in hypothalamus. Table 4 showed a significant decrease in total thiol in cortex, hippocampus(P<0.001) and hypothalamus (P<0.01) in G3 when compared with G1. In hippocampus there was a significant increase in G4 (P<0.01) and G5(P<0.001) when compared with G3. Table 5 showed a significant decrease in concentration of T. protein in cortex, hippocampus(P<0.01) and hypothalamus (P<0.001) in G3when compared with G1.

Table 1 : Serum concentrations of T3(ng/dl) , T4 (ng/dl) , TSH(ng/dl) and calcium (mmol/L) in G1 (control) G2 (folic acid) G3 (hyperthyroid) G4 (co-treated) G5 (post treated). Results expressed as mean ±SEM , n=6 .

Groups / Tests	G1	G2	G3	G4	G5
T3	32.72±1.0[a]	33.02±1.51[d]	94.34±2.09[a,d,g]	77.78±1.27[a,d,g,j]	48.5±2.63[a,d,g,j]
T4	3.43±0.15[a,b]	3.41±0.21[d,e]	6.14±0.36[a,d,g]	4.64±0.16[b,e,g]	3.75±0.16[g]
TSH	1.37±0.06[a]	1.23±0.07[d]	0.035±0.01[a,d]	0.075±0.01[a,d]	0.17±0.03[a,d]
Calcium	10.45±0.39[b]	10.68±0.45[f]	11.92±0.36[b,f]	11.31±0.28	11.2±0.21

The significant of difference was analyzed by one –way ANOVA and Tukey test (compare all pairs of columns)using a computer program (Graph Pad Instate software ,Inc)
- Groups having The same letters as a or d or g or j or m were significant at p≤ 0.001
- Groups having The same letters as b or e or h or k or n were significant from each at p≤ 0.01
- Groups having The same letters as c or f or i or l or o were significant from each at p≤ 0.05

Table2: concentration of catalase (mmol/ min/g) in brain tissues in G1 (control) G2 (folic acid) G3 (hyperthyroid) G4 (co-treated) G5 (post treated) . Results expressed as mean ± SEM , (n=6).

Groups / Tissues	G1	G2	G3	G4	G5
Cortex	0.91 ± 0.04[a]	0.98 ± 0.03[d,e]	1.93 ± 0.08[a,d,i,g]	1.29 ± 0.11[a,e,i]	1.25 ± 0.07[g]
Hypothalamus	0.51 ± 0.04[a]	0.55 ± 0.04[d,e]	1.1 ± 0.07[a,d,i,g]	0.87 ± 0.04[a,e,i]	0.65 ± 0.06[g]
Hippocampus	0.55 ± 0.03[a,c]	0.54 ± 0.02[d,f]	1.01 ± 0.07[a,d,i,h]	0.77 ± 0.04[c,f,i]	0.71 ±0.07[h]

The significant of difference was analyzed by one –way ANOVA and Tukey test (compare all pairs of columns)using a computer program (Graph Pad Instate software ,Inc)
- Groups having The same letters as a or d or g or j or m were significant at p≤ 0.001
- Groups having The same letters as b or e or h or k or n were significant from each at p≤ 0.01
- Groups having The same letters as c or f or i or l or o were significant from each at p≤ 0.05

Table 3: MDA concentration (nmol/g tissue) in brain tissues in G1 (control) G2 (folic acid) G3 (hyperthyroid) G4 (co-treated) G5 (post treated) . Results expressed as mean ± SEM , (n=6).

Groups / Tissues	G1	G2	G3	G4	G5
Cortex	17.37 ± 0.69[a,b]	19.58 ± 1.0[e]	25.67 ± 1.39[a,e]	23.4 ± 1.49[a]	23.9 ± 0.77[b]
Hypothalamus	12.22 ± 0.71[a,b]	13.04 ± 0.78[d,e]	24.36 ± 1.18[a,d,i]	21.66 ± 1.34[a,d]	19.34 ± 1.11[b,e,i]
Hippocampus	13.5 ± 0.94[b]	12.4 ± 0.79[e,f]	19.8 ± 1.0[b,e]	17.8 ± 1.6[f]	16.7 ± 1.4

The significant of difference was analyzed by one –way ANOVA and Tukey test (compare all pairs of columns)using a computer program (Graph Pad Instate software ,Inc)
- Groups having The same letters as a or d or g or j or m were significant at p≤ 0.001
- Groups having The same letters as b or e or h or k or n were significant from each at p≤ 0.01
- Groups having The same letters as c or f or i or l or o were significant from each at p≤ 0.05

Table 4 : Total thiol concentration (m mol \ g tissue). in brain tissues in G1 (control) G2 (folic acid) G3 (hyperthyroid) G4 (co-treated) G5 (post treated) . Results expressed as mean ± SEM , (n=6).

Groups / Tissues	G1	G2	G3	G4	G5
Cortex	1.37±0.07[a.c]	1.35±0.06[d,f]	0.92 ±0.05[a,d]	1.09±0.07[c,f]	1.16±0.05
Hypothalamus	1.71±0.36[b]	1.68±0.4[e]	1.03±0.19[b,e]	1.33±0.21	1.53±0.32
Hippocampus	1.97±0.12[a]	1.92±0.14[d]	0.99±0.12[a,d,g,h]	1.62±0.13[h]	1.86±0.09[g]

The significant of difference was analyzed by one –way ANOVA and Tukey test (compare all pairs of columns)using a computer program (Graph Pad Instate software ,Inc)
- Groups having The same letters as a or d or g or j or m were significant at p≤ 0.001
- Groups having The same letters as b or e or h or k or n were significant from each at p≤ 0.01
- Groups having The same letters as c or f or i or l or o were significant from each at p≤ 0.05

Table 5 : Total protein concentration (mg/g) in brain tissues in brain tissues in G1 (control) G2 (folic acid) G3 (hyperthyroid) G4 (co-treated) G5 (post treated) . Results expressed as mean ± SEM , (n=6).

Groups / Tissues	G1	G2	G3	G4	G5
Cortex	$25.6 \pm 0.97^{b,c}$	$26.5 \pm 0.89^{d,e}$	$20.8 \pm 0.62^{b,d}$	$21.4 \pm 0.78^{b,d}$	$22.3 \pm 0.47^{c,e}$
Hypothalamus	$27.8 \pm 0.57^{a,b}$	$27.0 \pm 0.72^{e,f}$	$21.0 \pm 0.79^{a,e}$	$22.3 \pm 1.25^{b,f}$	$22.4 \pm 1.15^{b,f}$
Hippocampus	$24.8 \pm 0.66^{b,c}$	23.55 ± 1.26^{f}	$19.35 \pm 0.86^{b,f}$	$20.0 \pm 0.67^{c,f}$	$20.8 \pm 1.16^{c,f}$

The significant of difference was analyzed by one –way ANOVA and Tukey test (compare all pairs of columns) using a computer program (Graph Pad Instate software ,Inc)

- Groups having The same letters as a or d or g or j or m were significant at $p \leq 0.001$
- Groups having The same letters as b or e or h or k or n were significant from each at $p \leq 0.01$
- Groups having The same letters as c or f or i or l or o were significant from each at $p \leq 0.05$

4. DISCUSSION

It is known that thyroid hormones regulate the energy metabolism of most tissues including liver, brain and heart. It is clear that thyroid hormones accelerate the basal metabolic rate and oxidative metabolism. The present study was designed to investigate the role of thyroid hormone on rat brain and the effect of hyperthyroidism on it, also to detect the effect of folic acid on the hyperthyroid state. For estimation of the improving effect of folic acid on the hyperthyroid state, we regularly determined the concentrations of serum T3, T4 and TSH. Results showed that serum T3 and T4 levels were increased and serum TSH levels were decreased in hyperthyroid group without treatment indicating the induction of hyperthyroid state. In the present study, we noticed that the effect of thyroxin on T3, T4 and TSH was reversed in rats when the treatment was withdrawn after 4 weeks (G5) and the concentrations of the T3, T4 and TSH tend to be nearing normal levels of the control. Also, serum T3 and T4 levels in co treated - and post-treated hypothyroid rats with folic acid were significantly decreased when compared with the hyperthyroid group which explained by the decreased production of T3 from the thyroid gland that minimizes TSH feedback inhibition mechanism which in turn leading to an increase in its secretion by the anterior pituitary gland; this result coincides with studies of (Shibutani et al., 2009).

Hyperthyroidism is characterized by accelerated bone turnover, which is caused from direct stimulation of bone cells by high thyroid hormone concentrations **(**Mundy et al., 1976 ; Abu et al., 1997). Many studies have also reported hypercalcemia in thyrotoxicosis. (Baxter and Bandy,1966) have reported prevalence of hypercalcemia to be around 23% in hyperthyroidism. In another series, percentage of hypercalcemia in thyrotoxicosis state varied between 5 & 27 % and this match with our study which showed a significant increase in the concentration of calcium in hyperthyroid group (G3). Recovering from hyperthyroidism causes insignificant decrease in the concentration of calcium,(G3).

It had been suggested that the TH influence the ROS steady-state and REDOX environment, while hyperthyroidism enhances the ROS production that affect the ROS steady-state and changes the REDOX environment causing cell damage. Because TH modulates many functions, so if TH levels changed, many cellular processes should be changed, leading to modifications in the REDOX environment. As the major effect of TH is the control of the basal metabolic rate, a hyper metabolic state produces a modification of the REDOX environment (Venditti and Di Meo, 2006). It is well-known that a higher concentration of T3 level, a hyper metabolic state, causes calorigenesis in two different ways. The first is a short-term signaling mechanism with the allosteric activation of cytochrome-C oxidase and the second is a long-term pathway producing nuclear and mitochondrial gene transcription through T3 signaling, thus stimulating basal thermogenesis (Oppenheimer et al., 1994). This last mechanism causes the synthesis of the enzymes involved in energy metabolism and the components of the respiratory-chain apparatus, leading to a higher capacity of oxidative phosphorylation (Soboll, 1993 and Videla, 2000). These two pathways are responsible for the increased cellular respiration which is caused by the hyperthyroid state. When the balance between oxidant and antioxidants is disturbed the cellular damage occurs and the antioxidant system does not balance the oxidants, thus altering the ROS steady-state level (Lushchak, 2011). The increase in catalase enzyme activity affording an increased antioxidant protection can be assumed as an incomplete compensatory mechanism which improved by our results that showed a significant increase in hyperthyroid group when compared with control group in all studied tissues ,these results are compatible with that of (Boveris et al., 1980 ; Fraga et al., 1987). Recovering from hyperthyroidism leads to a

decrease in oxidative stress which causing a decrease in catalase enzyme activity.

The biological significance of the oxidative modification of proteins in T3-caused oxidative stress can be visualized on two levels; 1) loss of protein function and 2) increased protein degradation which explain the significant decrease of total protein in brain tissues in hyperthyroid group (G3) when compared with control group (G1) . These findings are in accordance with those of (Tapia et al., 2010) who find that thyrotoxicosis in mammals results in the stimulation of both synthesis and degradation of protein, with a predominance of degradation, which is showed by the increase in protein catabolism, negative nitrogen balance, and the loss of protein from the muscles and body tissues. Treating by folic acid showed insignificant increase in total protein contents (G4 and G5) and this may be due to apoptosis of the brain protein tissue.

It has been well documented that thyroid dysfunction increases lipid peroxidation reactions (evidenced by the elevated MDA levels) and reactive oxygen species (Messarah et al., 2007) and these match with the present study as there was significantly increase in MDA of brain tissues of hyperthyroid group (G3) when compared to the control group (G1) and folic acid group (G2).

The hyperthyroid state leads to imbalance between oxidant and antioxidants causing cellular damage and the antioxidant system do not balance the oxidants leading to a decrease in T. thiol concentration. The significant increase in total thiol of brain tissues in co-treatment group (G4) as compared to hyperthyroid group (G3) in hippocampus was explained by the role of folic acid in restoring oxidant balance without restoration of euthyroid state as presented here. However in post-treated group (G5), there is a significant increase in total thiol as compared to hyperthyroid group (G3), 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 (Ibrahim et al., 2011).

5. CONCLUSION

This study showed that hyperthyroidism increase oxidative stress in different brain tissues and leads to a number of damages, while treating with folic acid and restoring euthyroid state cause improvement in oxidative stress parameters and restoring normal neuronal structures.

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