

Levothyroxine replacement therapy with vitamin E supplementation prevents the oxidative stress and apoptosis in hippocampus of hypothyroid rats

Yanyun GUO¹, Si yuan WAN², Xing ZHONG¹, Ming Kui ZHONG³, Tian Rong PAN¹

¹ Department of Endocrinology, The Second Affiliated Hospital of Anhui Medical University, Hefei, People's Republic of China

² Department of Endocrinology, The 82nd Hospital of the People's Liberation Army, HuaiAn, People's Republic of China

³ Department of Physiology, Institute of Basic Medical Sciences of Anhui Medical University, Hefei, People's Republic of China

Correspondence to: Tian Rong Pan,
 Department of Endocrinology,
 The Second Affiliated Hospital of Anhui Medical University,
 Hefei, 230601, People's Republic of China.
 TEL: +86-0551-63869568; E-MAIL: ptr1968@163.com

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Abstract

OBJECTIVE: To examine the effect of levothyroxine (L-T4), vitamin E or both on oxidative stress status and hippocampal apoptosis in a propylthiouracil (PTU)-induced hypothyroid rat model.

METHODS: Sprague-Dawley rats were randomly divided into five groups: Control, PTU+PTU+L-T4+PTU+Vit E, PTU+Vit E+L-T4. In each group we assessed levels of serum triiodothyronine (T3), tetraiodothyronine (T4), thyroid stimulating hormone (TSH), hippocampus cellular apoptosis index (AI), hippocampus nicotinamide adenine denucleotide hydrogen (NADPH)oxidase and superoxide dismutase (SOD).

RESULTS: 1) Compared with the control group, NADPH oxidase levels were significantly increased, and SOD levels were significantly reduced in the PTU groups ($p < 0.05$). 2) Compared to the PTU group, SOD levels were significantly increased in the PTU+Vit E and PTU+L-T4+Vit E group ($p < 0.05$). NADPH oxidase levels were significantly decreased in the PTU+L-T4, PTU+Vit E and PTU+L-T4+Vit E group ($p < 0.05$). 3) Compared with the control group, hippocampus AI increased significantly in the PTU group ($p < 0.05$). Compared with the PTU group, hippocampus AI was significantly reduced in the PTU+L-T4 group and PTU+L-T4+Vit E group ($p < 0.05$). 4) Hippocampus AI was positively correlated with NADPH oxidase expression levels in hippocampus tissue ($r = 0.644$, $p < 0.01$).

CONCLUSION: Levothyroxine replacement therapy combined with vitamin E reduces hippocampus AI by improving oxidative stress. This study suggested that the mechanisms of hippocampus tissue injury in a hypothyroid rat model is related to hippocampus apoptosis from increased oxidative stress.

Abbreviations:

PTU	- propylthiouracil
L-T4	- levothyroxine
T3	- triiodothyronine
T4	- tetraiodothyronine
TSH	- thyroid stimulating hormone
AI	- apoptosis index
NADPH	- nicotinamide adenine denucleotide hydrogen
SOD	- superoxide dismutase
MDA	- malondialdehyde
HRP	- horseradish peroxidase
GAPDH	- glyceraldehyde phosphate dehydrogenase
TUNEL	- terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
PVDF	- polyvinylidene fluoride
ROS	- reactive oxygen species

INTRODUCTION

Many studies have found that the increase in reactive oxygen species during both hypothyroid and subclinical hypothyroid states lead to oxidative stress and lipid peroxidation in several organs such as liver, heart, skeletal muscles, and brain (Torun *et al.* 2009; Santi *et al.* 2012). The central nervous system features of hypothyroidism include mental retardation, various cognitive and memory deficits derived from the hippocampus, an important organ in the maintenance of memory. Many studies have shown that increased oxidative stress in the hippocampus of hypothyroid rats results in nerve injury (Cano-Europa *et al.* 2008; Hosseini *et al.* 2010; Cattani *et al.* 2013). Previous studies have reported that increased oxidative damage significantly reduces spatial learning in the hippocampus of hypothyroid rats and suggested that levothyroxine replacement therapy with vitamin E supplementation might ameliorate cognitive deficit through a decrease in oxidative stress (Pan *et al.* 2012). The present study aimed to evaluate the levels of oxidative stress and apoptosis in the hippocampus, before and after L-T4 and/or Vit E replacement therapy in a PTU-induced hypothyroid rat model. Further, we examined the relationship between hippocampal cell apoptosis and cognitive defect to provide a theoretical basis for clinical treatment of hypothyroidism.

MATERIALS AND METHODS**Materials**

PTU, L-T4, and Vit E were obtained from Sigma Chemical Co (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG was purchased from Santa-Cruz Biotechnology Inc. (Santa Cruz California, CA, USA); HRP-labeled anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) single resistance was purchased from Kangchen Biotechnology Inc. (Shanghai, China); Terminal deoxynucleotidyl transfer-

ase-mediated dUTP nick end labeling (TUNEL) assay kit was obtained from Roche Molecular Biochemical Company.

Animal treatment and sample collection

Forty six male Sprague-Dawley rats, weighing 215±20 g, were purchased from Laboratory Animal Center of Nanjing Medical University (Nanjing, China), animal production license: SCXK (Su) 2008-0004. Protocols for animal care, maintenance, and experiments were approved by the Institutional Animal Ethics Committee. For the animal adaptability, all rats had been fed for one week before the experiment. Rats were randomly divided into five groups, a control group (n=8) and other four groups (n=38). Rats in the control group were fed standard diet and drinking water *ad libitum*. Rats in the treated groups were treated with 0.05% (w/v) PTU in drinking water for four weeks. After completion of this time period, rats were randomly divided into four groups and administered PTU: Animals in the PTU group (n=11) continued to receive PTU for another 28 days; the PTU+L-T4 group (n=9) and PTU+L-T4+Vit E groups (n=9) were given L-T4 (2 µg/100 g per day in 1 mL vehicle) at 8:00 AM, Rats of other groups were treated with saline solution for 28 days; Animals in the PTU+Vit E group (n=9) and PTU+L-T4+Vit E group were given Vit E (20 mg/100 g per day in 1 mL vehicle) at 6:00 PM, and rats of other groups were treated with vegetable oil for 28 days. T4 and Vit E were administered by intragastric route.

By the end of the study period (8 weeks), some rats were died of misoperation during intragastric administration. So there are 8 rats in control group, 10 rats in PTU group, 7 rats in PTU+L-T4 group, 8 rats in PTU+Vit E group and 8 rats in PTU+L-T4+Vit E group respectively. Rats were carefully weighed in the morning before killed by chloral hydrate anesthesia (0.3 mL/100 g body weight, i.p.). Blood samples were obtained by abdominal aorta puncture and centrifuged at 3000 rpm for 10 min. Serum was separated and stored into EDTA-containing tubes at 4°C until assessment of T3, T4 and TSH concentrations.

Rats were sacrificed by decapitation after blood collection. The hippocampus were quickly removed on ice, the left hippocampus were stored at -80°C for Western blot analysis, The right hippocampus were placed in 10% neutral buffered formalin prior to embedding the samples in paraffin wax.

Measurement of serum thyroid hormone levels

Serum T3, T4, and TSH concentrations were measured by radioimmunoassay, using reagents supplied from North Institute of Biological Technology (Beijing, China), as previously described (Yang *et al.* 2012). The detection ranges of the assay were 0.2–4 nmol/L for T3, 20–320 nmol/L for T4, and 0.15–60 µIU/mL for TSH. Blood samples were obtained between 09:00 and 11:00 AM in a randomized fashion.

Measurement of hippocampal expression of NADPH oxidase and SOD content

Samples were collected and loaded in a gel for Western blot analysis, and proteins were separated via electrophoresis (15% stacking gel at 25 mA, 4% separating gel at 40 mA). GAPDH, SOD and NADPH oxidase were then transferred to polyvinylidene fluoride (PVDF) membranes (GAPDH and SOD half dry transfer 15 V, 2h; NADPH oxidase wet transfer 80 V, 30 min). Blots were incubated with the corresponding primary antibody (gp91phox, 1:200) at 4 °C on a shaker overnight, followed by incubation with HRP-labeled rabbit anti-goat IgG (1:80000). For internal reference, HRP-labeled anti-GAPDH antibody (1:10 000) was incubated for 1.5 hours at room temperature. Electrochemiluminescence detection solution was added onto each PVDF membrane, then developed and fixed in a dark room. The ratio of NADPH oxidase and SOD band intensity compared to GAPDH band intensity were measured among the different groups, which were used to estimate protein expression level indirectly.

Determination of hippocampal AI

A TUNEL assay was used to identify apoptotic cells. Following fixation, dehydration, and embedment, a graded ethanol series the specimens were incubated with proteinase K in a moist chamber at 37 °C for 15 minutes, and were washed twice in PBS, each for 3 minutes, and patted dry on the filter paper. Finally, specimens were incubated with 50 µL TUNEL reaction mixture in a moist chamber at 37 °C in dark for 120 minutes. The sections were observed under fluorescence microscopy (green), and computational modeling was conducted to analyze signal transduction. The sections were exposed to 3,3'-diaminobenzidine reagent, counter-stained with hematoxylin, dehydrated with ethanol of increasing grade, and mounted. Optical microscopy was used to detect positive signal for apoptosis, as indicated by brown or yellow color in the nucleus. Positive cells/total cells were calculated in 10 non-overlapping fields (10×50 microscope) from each slide. AI=(positive cells/total number of cells)×100. Hippocampal cells AI was the mean value.

Statistics

Data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and presented as mean ± standard deviation. Inter-group differences were assessed by a one-way analysis of variance. The parameters of oxidative stress and hippocampus cellular apoptosis index were used to perform linear correlation analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of L-T4 or/and Vit E on thyroid hormone status in PTU-treated hypothyroid rats

Serum T3 and T4 levels were significantly decreased, whereas TSH levels were significantly increased in rats

treated with PTU and PTU+Vit E compared with the control group ($p < 0.05$). Serum T3 and T4 levels were significantly increased but TSH levels were significantly decreased in rats treated with PTU+L-T4 and PTU+L-T4+Vit E group compared with the PTU group ($p < 0.05$; Figure 1 a–c).

Effects of L-T4 or/and Vit E on NADPH oxidase and SOD expression levels in the rat hippocampus

NADPH oxidase levels (Figure 2 a–b) in the PTU groups were significantly higher than the control

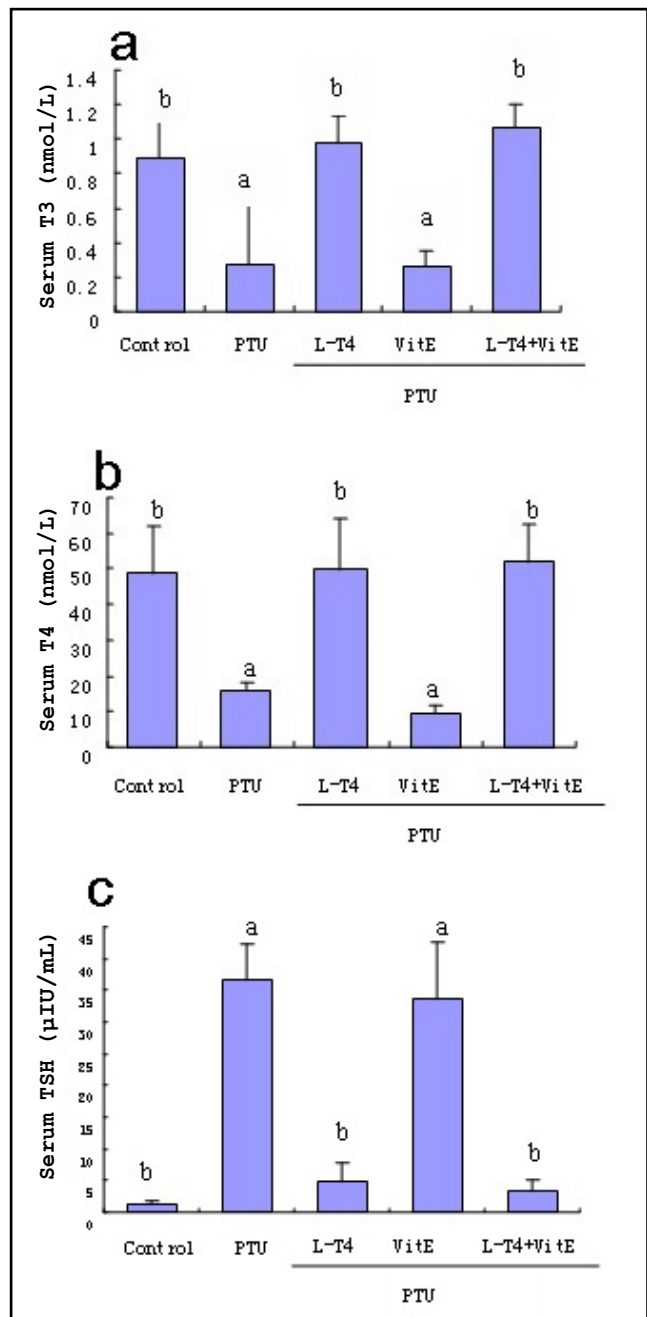


Fig. 1. Comparison of serum thyroid hormone levels in rats treated with PTU, PTU + L-T4, PTU + Vit E, and PTU + Vit E + L-T4. Data are expressed as mean ± SD. The analysis of data was done by one-way ANOVA, $a p < 0.05$ compared to the control group; $b p < 0.05$ compared to the PTU group.

group ($p < 0.05$), while NADPH oxidase levels were significantly decreased in PTU+L-T4, PTU+Vit E and PTU+L-T4+Vit E groups compared with the PTU group ($p < 0.05$).

SOD levels (Figure 2 a-c) in the PTU and PTU+L-T4 groups were significantly lower than the control group ($p < 0.05$). However, SOD levels were significantly increased in PTU+Vit E and PTU+L-T4+Vit E group compared to the PTU group ($p < 0.05$).

Effects of L-T4 or/and Vit E on AI in the hippocampus of rats

AI (Figure 3 a-e) in the PTU and PTU+Vit E group were significantly higher than the control group

($p < 0.05$). The AI was significantly lower in PTU+L-T4 and PTU+L-T4+Vit E groups than the PTU group ($p < 0.05$; Figure 3 f).

Correlation analysis between AI of the hippocampal cells and expression of NADPH oxidase and SOD

There was a significant positive correlation between the AI of the hippocampal cells and NADPH oxidase expression in the hippocampus ($r = 0.644$, $p < 0.01$). Analysis shows that there is no statistically significant correlation between AI and expression of SOD of hippocampus ($r = -0.316$, $p = 0.064$).

DISCUSSION

Thyroid hormones are essential for brain development and function. Adult hypothyroid patients show cognitive deterioration, memory impairment, and depression symptoms (Boelaert *et al.* 2005; Chen *et al.* 2012; Corte's *et al.* 2012). Insufficient thyroid hormone during development alters hippocampal synaptic function and impairs behavioral performance of hippocampal-dependent learning and memory tasks that persist in euthyroid adult animals (Rivas *et al.* 2007). Desouza (Desouza *et al.* 2005) demonstrated that adult-onset hypothyroidism significantly decreases hippocampal neurogen in the rat brain. Mal-Soon (Shin MS *et al.* 2013) found that doublecortin protein expression in the hippocampal dentate gyrus, brain-derived neurotrophic factor and tyrosine kinase B were significantly decreased in the hypothyroid rats. Hypothyroidism results in reduced levels of protein expression or cytokines involved in the formation migration and differentiation and synaptic plasticity of new neurons in the hippocampus. Moreover hypothyroidism increases the numbers of caspase-3-positive cells in the dentate gyrus, indicating that hypothyroidism can induce dentate gyrus neuron apoptosis. A preliminary study by Pan *et al.* (2012) showed that PTU-induced hypothyroid rats exhibits reduced spatial learning ability and increased oxidative stress. In present study, we observed that the hippocampal oxidative stress and AI were significantly increased in the hypothyroid rat, indicating that neuronal apoptosis might be the mechanism of learning disability. Our study also found that there is a significant positive correlation between hippocampal AI and NADPH oxidase levels, this suggested that hippocampal neuronal apoptosis resulting in excessive lipid peroxides and damaged hippocampal tissue might cause abnormal neural activity. The mechanism underlying increasing hippocampal neuronal apoptosis in hypothyroid rats appears to be an imbalance between oxidase enzyme and antioxidant enzyme expression.

Oxidative stress can put the body in a state of vulnerability and also enhance the toxic effects of pathogenic factors that can induce genetic mutations (Beck 1999). It can cause occurrence of various diseases, and is associated with cell apoptosis. The increase in the levels of

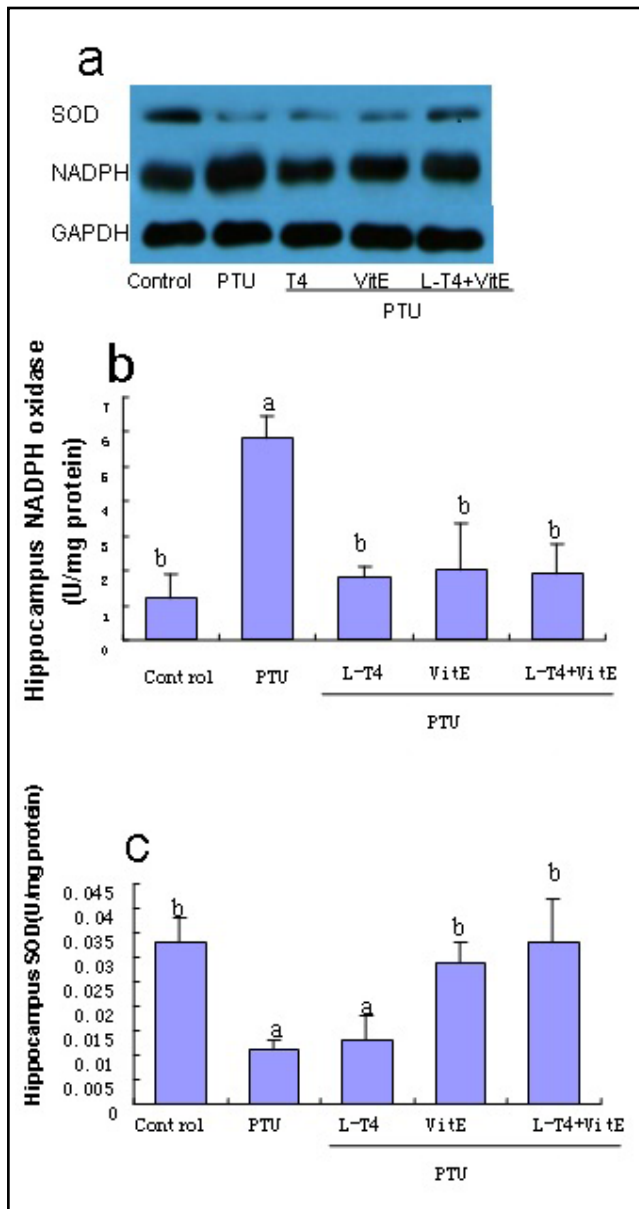


Fig. 2. Comparison of hippocampus NADPH oxidase and SOD levels in rats treated with PTU, PTU + L-T4, PTU + Vit E, and PTU + Vit E + L-T4. Data are expressed as mean \pm SD. The analysis of data was done by one-way ANOVA, ^a $p < 0.05$ compared to the control group; ^b $p < 0.05$ compared to the PTU group

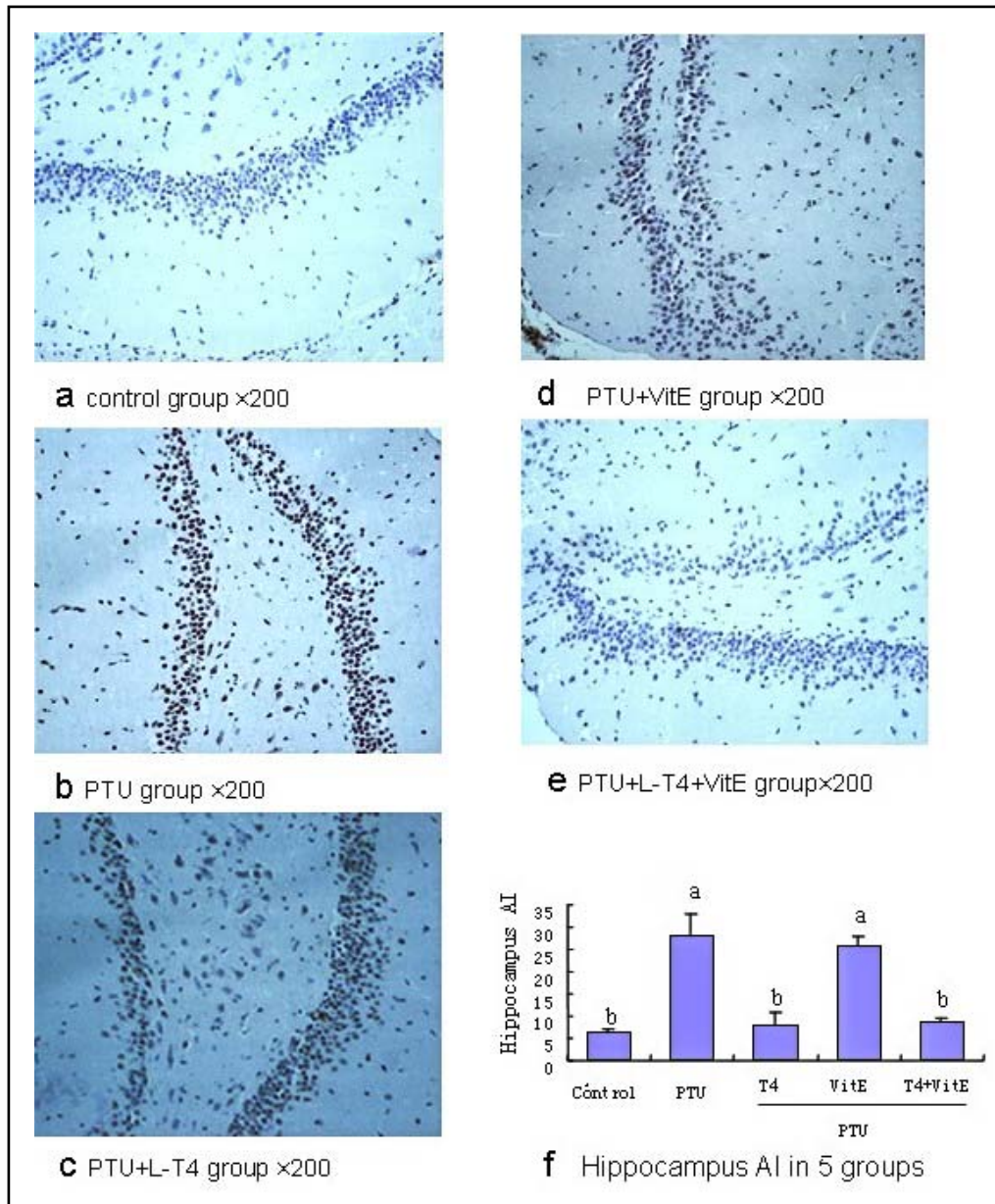


Fig. 3 a–e: TUNEL staining of hippocampus levels in rats treated with PTU, PTU + L-T4, PTU + Vit E, and PTU + Vit E + L-T4. f: Hippocampus apoptotic index in 5 groups

endogenous or exogenous active oxygen can result in cell apoptosis. The mechanisms involved in oxidative stress mediated cell apoptosis might be endoplasmic reticulum stress – Tabas *et al.* 2011, mitochondrial pathway (Deryabina *et al.* 2014), and activation of mitogen activated protein kinase (Inoshita *et al.* 2002), nuclear transcription factor κ B (Anqkeow *et al.* 2002) or caspases (Carmody *et al.* 2000).

Activation of NADPH oxidase, a kind of peroxidases causes excessive production of ROS and ROS-mediated oxidative stress damage (Bedard *et al.* 2007). NADPH oxidase contributes to many pathological processes. Enhanced activity of the NADPH oxidase may result in wide pathological disorder, including cardiovascular (Csanyi *et al.* 2009) and neurodegenerative diseases (Choi *et al.* 2005; Zekry *et al.* 2003). Studies have demonstrated a synaptic localization of NADPH oxidase

protein expression in the hippocampus (Tejada-Simon *et al.* 2005; Massaad *et al.* 2011). Nair *et al.* have shown that hippocampal apoptosis and neurobehavioral impairments were mediated by excessive NADPH oxidase activity (Nair *et al.* 2011). SOD also plays an important role in the body's antioxidant system and is involved in the protection of organism and anti-aging (Buettner 2011; Holley *et al.* 2010). In present study, the levels of SOD in the hypothyroid rat hippocampus were significantly reduced, while the NADPH oxidase expression was significantly increased. These findings indicate that oxidative stress and antioxidant defense system might be involved in neural cell damage caused by hypothyroidism.

Vitamin E, a fat-soluble antioxidant, can be incorporated into cell membranes, and can protects the cell membrane against oxidative damage (Alzoubi *et*

al. 2013; Zakharova *et al.* 2013). Studies have found that vitamin E has inhibitory effect on cell apoptosis (Wambi *et al.* 2008; Serbecic *et al.* 2005). In present study, we observed significant effect of vitamin E on antioxidative stress and found that the level of NADPH was positively related to the AI of hippocampus. However, vitamin E has no obvious improvement effect on hippocampus cell apoptosis. This observation could be confounded by the small sample size of our study. Thus, further studies with large sample size are warranted to test whether vitamin E inhibits apoptosis, at least it can speculate that vitamin E can improve the level of NADPH which is beneficial to inhibiting apoptosis.

Alzoubi *et al.* (2009) have found that thyroxine treatment reverses hypothyroidism-induced impairment of hippocampus-dependent cognition, and late-phase long-term potentiation (L-LTP), probably by restoring the levels of signaling molecules important for electrophysiological and molecular changes. Thyroid hormone has shown to be involved in the maintenance of hippocampal neurons, even in the adult rat, suggesting that thyroid hormone acts as a survival factor for neurons and exhibits different physiological functions (Alva-Sanchez *et al.* 2009). T4 can prevent the activation of apoptosis pathway, while T3 is involved in regulating cell proliferation and differentiation. Our study showed that levothyroxine significantly increases the levels of this hormone but decreases the level of NADPH oxidase and hippocampal AI at the same time, indicating that the thyroid hormone might inhibit cell apoptosis through improvement of oxidative stress.

In the present study, we found oxidative damage and apoptosis in the hippocampus of hypothyroid rats and showed that the level of NADPH was positively related to AI of hippocampus. This suggests that oxidative stress may be involved in the pathogenesis of apoptosis in the hippocampus of hypothyroid rats. Moreover, levothyroxine replacement therapy concurrently reduced NADPH oxidase levels and hippocampal AI. However, Vit E supplementation had no direct effect on apoptosis of the hippocampus, although it reduces the level of oxidative stress in the hippocampus. Also, previous studies suggested that Vit E supplementation might ameliorate cognitive impairment by reducing of oxidative stress. Together, we advocate that thyroid hormone replacement therapy should be combined with Vit E.

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