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ORIGINAL PAPER

Hippocampal Up-Regulation of Apolipoprotein D in a Rat Model of Maternal Hypo- and Hyperthyroidism: Implication of Oxidative Stress

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Abstract

Thyroid disorders impair various functions of the hippocampus where thyroid hormone receptors are localized in the brain. Hyper and hypothyroidism are associated with large changes in brain oxidative stress. Apolipoprotein D (APOD) is a conserved glycoprotein that increased in response to oxidative stress in the brain and has been suggested function as an antioxidant in the brain. Thus, the goal of this work was to explore the effect of maternal hypo- and hyperthyroidism on the *Apod* expression in the pup's brain regarding changes in oxidative stress. For induction hypo and hyperthyroidism in adult female rats, 100 ppm propylthiouracil (PTU) and 8 ppm levothyroxine administrated 1 month before copulation to the week 3 after delivery in drinking water. The hippocampal region of rat pups was isolated and used for immunohistochemistry and quantitative RT-PCR on postnatal day (PND)5, PND10 and PND20. Results revealed that APOD over-expressed in both hypo- and hyperthyroid groups on PND5, PND10, and PND20. There was a proportional increase between the *Apod* expression and oxidative stress in the hyperthyroid group but not the hypothyroid in different days. Regarding the wide functions of thyroid hormones, oxidative stress does not suggest to be the only mechanism that involves *Apod* gene expression in thyroid disturbances.

Keywords Apolipoproteins D · Hyperthyroidism · Hypothyroidism · Oxidative stress · Hippocampus · Thyroid hormones

Abbreviations

THs	Thyroid hormones
Apod	Apolipoprotein D mRNA
APOD	Apolipoprotein D protein
PND	Postnatal day
PTU	Propylthiouracil
TAC	Total antioxidant capacity
MDA	Malondialdehyde
OS	Oxidative stress
TBA	Thiobarbituric acid
PBS	Phosphate buffered saline
TBS	Tris-buffered saline
BSA	Bovine serum albumin
DAB	3,3-Diaminobenzidine
HRP	Horseradish peroxidase
DG	Dentate gyrus

Introduction

Thyroid hormones (THs) have an important role in prenatal and early postnatal development [1]. In the process of brain development, thyroid hormones affect a variety of processes, including neurogenesis, glial evolution, and proliferation of dendritic outspreads. Defects in thyroid hormones, even in a short period, lead to irreversible brain damage [2]. In the brain, it has been shown that THs to affect cell maturation in areas with considerable postnatal neurogenesis, including the hippocampus [3]. The molecular mechanism of the consequences of THs deficiency on evolution during pregnancy is unknown, but recently Katherine's study on pregnant rats receiving 10 ppm PTU in five days periods caused heterotopia in the pups. They have shown that prenatal THs insufficiency changes neural progenitor cells migration [4].

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In thyroid disorders, has reported biochemical and morphological abnormalities in the hippocampus [5]. Studies show mild-to-moderate prenatal hypothyroidism can significantly reduce glutamine, glycine, GABA, and NMDA receptor subunits in the hippocampus. It has been shown that mild hypothyroidism irreversibly reduces the production of glutamine. The deficiency of thyroid hormones during pregnancy reduces granular cell in the *dentate gyrus* (DG) in the hippocampus's formation. THs insufficiency reduces the size of DG`s mossy fibers and the number of synapses developed between these fibers and pyramidal cells of the *cornu amonis* (CA3) [6, 7]. In addition, Neonatal hypothyroidism leads to a significant reduction of CA1 pyramidal cells [8]. A lack of thyroid hormones causes synaptic disorders. Perhaps the reduction in protein substrates involved in synaptic plasticity impairs the neurophysiological function of the hippocampus [9]. On the other hand, hyperthyroidism causes a significant decrease in the density of apical dendritic spines in the CA1 region [10]. During the postnatal developmental period of the brain, thyroid hormone imbalances can lead to impairments in differentiation, apoptosis, and proliferation of glial cell types [11].

The abnormalities associated with thyroid disorders may occur due to oxidative stress-induced damage [12]. High levels of thyroid hormones can cause thyrotoxicosis, which in turn, expedites metabolic reactions and increases oxygen consumption and oxidative reactions. The malfunction of the respiratory chain in the mitochondria and metabolic disorders caused by hypothyroidism can also elevate oxidative stress [13]. Cano-Europa et al. investigation revealed that the reactive oxygen spices (ROS) and lipid peroxidation (LPO) rise in the amygdala and hippocampus of hypothyroid rats. It has suggested oxidative damage to the lipids, proteins, and carbohydrates reduce the number of neurons in the CA3 region of the hippocampus and cause other neurological changes [14]. Studies established that hypothyroidism decreases glutathione (GSH) reserves in cortical and hippocampus cells [15]. Microtubule-related protein-2 (MAP-2) is essential for the formation of microtubules in neurons. It has been revealed that an interrelationship between oxidative stress and increased phosphorylation of this protein in the degeneration of neurons in the CA3 zone of the hippocampus [16]. It was indicated that PTU-induced hypothyroid rat had an increase in oxidative stress and an up to 70% MAP-2 down-regulation in CA1 and CA3 hippocampal regions and neural structures and synaptic transmission altered. In this situation, neural structures, and synaptic transmission altered [17].

Apolipoprotein D (APOD) is a multifunctional conserved glycoprotein from the lipocalin family that is expressed in mammalian tissues [18]. A growing body of evidence suggests that APOD acts as part of the organism's natural defense system against oxidative stress in degenerative brain disorders [19–21]. In fact, APOD is up regulated in a range of neurologic disorders associated with cellular stress [22]. With damage to the central nervous system, APOD acts as a neuroprotective agent. It has proposed several roles for APOD including the antioxidant role and transferring compounds such as arachidonic acid, cholesterol, and retinoic acid [23, 24]. It is reported that brain APOD increases in response to oxidative stress, enhancing LPO in the brain [25]. Studies have shown any cellular stress that accompanies growth arrest, for example, H_2O_2 and UV increase the APOD expression. Carmo's investigation showed in spite of lipopolysaccharide (LPS) increase, cell proliferation could also up regulate APOD. Other stressful conditions, such as temperature variations, osmotic pressure alteration, and contact with metals and drugs, like camptothecin, which is an apoptotic stimulant, does not influence the APOD expression [26]. Therefore, any stress status may start APOD expression. It has been proposed that APOD is a nonspecific response to different irritants and may be part of the body's antioxidant capacity [27].

Previous studies have shown there is a high density of thyroid hormone receptors in the hippocampus [28]. The APOD levels was higher in the hippocampus than in other regions of the brain of rats [29]. On the other hand, hyper and hypothyroidism are associated with brain oxidative stress. Thus, the goal of this work was to explain the effect of maternal hypo- and hyperthyroidism on the offspring's brain APOD level on postnatal days regarding changes in oxidative stress. In this work, we showed that APOD might play a role in the hippocampal function and development in thyroid hormone abnormalities.

Materials and Methods

Animals

In this study, we used white Wistar rats including 45 mature virgin females and 15 mature males (180 ± 25 g). We gained the animal rats from the breeding colony of Semnan University of Medical Sciences and housed in cages (12 h dark/light cycle, temperature 23 ± 1 °C) where they had access to food and water. The Ethics Committee of Semnan University of Medical Sciences accredited the study.

Experimental Design and Preparation of Serum and Tissue Samples

After a 4 week period of adaptation, we randomly distributed animals into the three groups of control, hypothyroid, and hyperthyroid. For the hypothyroid group (n = 10), maternal hypothyroidism induced by adding propylthiouracil or PTU (Sigma Co., USA) to drinking water (100 mg/L). The

treatment began on day 21 before pregnancy and continued until day 20 of lactation. We selected the PTU dose based on a previous study [30]. The control group (n = 10) only received water during this period. For the hyperthyroid group (n = 25), maternal hyperthyroid induced by adding levothyroxin (Aburaihan Co., Iran) to drinking water (8 mg/L) from day 21 before pregnancy until day 20 of lactation. To minimize pups' mortality, we determined the T4 dose experimentally.

We kept female rats in individual cages until the birth of the pups. Control, hyperthyroidism, and hypothyroidism groups continued to take water, levothyroxine, and PTU respectively. We designated the day of birth as postnatal day (PND0). Two pups were sacrificed from each dam and totally 15 pups from each group under deep anesthesia by decapitation on PND5, PND10, and PND20 (Fig. 1). The males were only used for breeding i.e. no experimental manipulations. To conduct the immunohistochemical testing of the $APOD^{+}$ cells in the hippocampus, brain tissues isolated, cleaned in ice-cold normal saline, and fixed in 10% formalin.

For lipid peroxidation (LPO) and total antioxidant capacity (TAC) assays in the brains of the offspring, the brains were removed and cleaned in ice-cold normal saline. We dissected the region containing the hippocampus from the right hemisphere before snap freezing and storage at − 70 °C. For quantitative real-time polymerase chain reaction (qRT-PCR) experiments, hippocampus regions were separated, cleaned in ice-cold normal saline, homogenized in ice-cold TRIzol solution, and were incubated at − 70 °C. We sacrificed rat dams under deep anesthesia. Blood samples were collected, and the serum was isolated and stored at − 70 °C.

Maternal Serum Total Triiodothyronine (TT3) Measurement

Serum TT3 measurements were performed using ELISA commercial kit according to the manufacturer's instructions (Dia plus Co., USA).

The Oxidative Stress Markers Measurement

Brain lysate was prepared in 1 mL of ice-cold phosphate-buffered saline (PBS) with a pH of 7.4. The homogenate samples were centrifuged at 13,000×*g* for 20 min. The supernatant was collected and used for the measurement of proteins, malondialdehyde (MDA), and TAC. Total protein was measured by the standard Bradford method [31]. The extent of lipid oxidation was determined by measuring MDA level. MDA level in the brain tissue was estimated by the thiobarbituric acid (TBA) method [32]. TCA was measured according to the method described previously [33]. In all the assays, the experimental values were normalized to protein concentration and results were expressed as µmol/mg protein.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For *Apod* mRNA analysis, total RNA was extracted from TRIzol-homogenized samples using RiboExTM (GeneAll Co., South Korea). The absorbance ratios of 260/280 nm were used to determine RNA purity. 2 µL total RNA was used for reverse transcription reactions with Prime Script TM RT reagent kit (Takara Co, Japan). The gotten cDNA were used as a template for qRT-PCR using SYBR Green (Ampliqon Co., Denmark). The amplifications were performed in an ABI-Step one plus-RT-PCR System. Quantitative PCR cycling program was 95 °C for 15 min, pursued by 40 cycles at 95 °C for 15 s, 60 °C

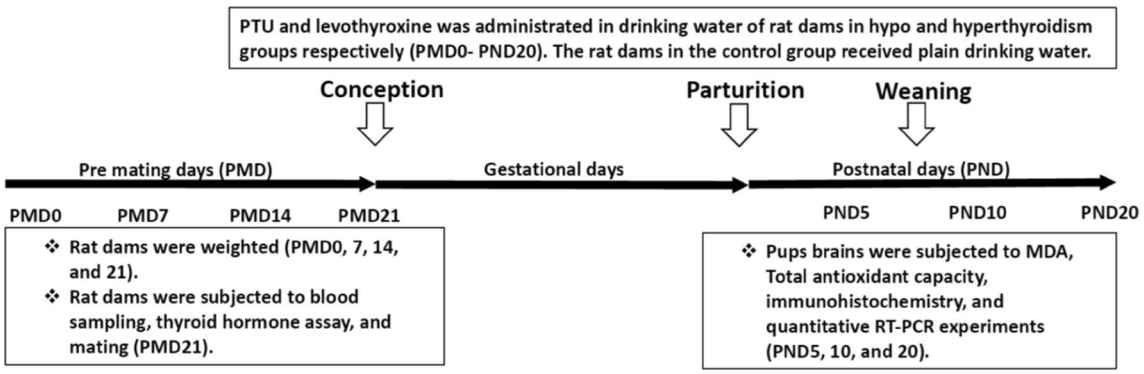

Fig. 1 Timeline of experiments

Table 1 Primers properties were used for qRT-PCR

Gene	Primer sequences[a]	Sequence accession no.	Amplicon length
Apod	GGAGAACGGAAACATCAAAGTG GGTGGCATCAACGAGAAGA	NM_012777.1	138 bp
Actb	CACACCCGCCACCAGTTCG ACCCATTCCCACCATCACACC	NM_031144.3	165 bp

[a]Upper sequences are forward primers

for 1 min, and a melting curve analysis at 96 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. We estimated changes in transcriptional expression with the ΔΔCt method. The values are expressed as the means of $2^{-\Delta\Delta Ct}$ [27]. We used the *Actb* as an internal control gene. The oligonucleotides were designed by Allele. ID 7.5 primer design software (Table 1).

Immunohistochemistry (IHC) Method for APOD$^+$ Cell Staining

For IHC study, the fixed brains were cut with a microtome (Feitz Co., Germany) at a thickness of 5 µm. We incubated the sections at 55 °C for 2 h and paraffin eliminated by soaking in xylene for 15 min for removal of paraffin. The samples were rehydrated by incubation in a graded alcohol set (3 min for each percentage) and washed for 3 min with Tris buffer. For quenching peroxidase activity, the sections were treated with 3% H_2O_2 in Tris-buffered saline (TBS) for 15 min. Then, the antigen retrieval stage was accomplished by incubating the prepared sections in boiling Tris buffer (pH 9.0, 98 °C, 20 min). The sections were then washed with TBS-tween-20 twice for 5 min.

To eliminate non-specific binding, the samples were incubated in a 1% bovine serum albumin (BSA) in TBS for 2 h at room temperature. The sections were incubated with 1/150 diluted anti-APOD antibody (ab187513; Abcam, USA). After overnight incubation at 4 °C, the sections were washed three times with TBS-Tween-20 for 5 min. Then, they were incubated with 1/100 diluted goat anti-rabbit secondary horseradish peroxidase (HRP) antibody (ab7090, Abcam, USA) at room temperature in the dark for 1 h and washed three times with TBS-tween-20 for 5 min. Then the tissue sections were incubated for 10 min at room temperature with DAB (ab94665, Abcam, USA). The stained slides were washed with distilled water for 5 min. The sections were counter stained with Hematoxylin & Eosin for 1 min, dehydrated, cleared, and mounted.

Statistical Analysis

Statistical analysis was carried out using SPSS software version 22. The results were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-test and are presented as means ± SEM. The $p < 0.05$ was considered as statistically significant.

Table 2 Effect of status thyroid on total triiodothyronine (TT3 ng/dL) concentration in serum of mothers at PND21

Groups	TT3 (ng/ dL), mean ± SEM	p value
Control (n = 5)	149 ± 7.42	
Hypothyroid (n = 5)	66.439 ± 6.016	< 0.001
Hyperthyroid (n = 5)	198.193 ± 10.12	< 0.01

Data are expressed as mean ± SEM. Hypo and hyperthyroid groups were compared versus control group

Results

Maternal Total Triiodothyronine (TT3) Concentration

Table 2 shows that administration of levothyroxine to rat dams significantly increased the level of serum total T3 compared with control group and administration of PTU significantly decreased the level of serum total T3 in hypothyroid mothers compared with control group.

The Effects of Treatment with Levothyroxine and PTU on Body Weight in Rat Dams

The weight of rat dams was also measured for confirmation of the models, in control, hypothyroid and hyperthyroid groups before starting treatment with the drug and three consecutive weeks after starting the medication. Analysis of the body weight in hypothyroid group showed that after 21 days of taking the PTU, the average of body weight increased, but the percentage of body weight gain was significantly lower than the weight gain percentage of the control group ($p < 0.01$). Taking the levothyroxine drug reduced the weight of mothers in the hyperthyroid group (Fig. 2 a, b).

Thyroid Dysfunction Increase Oxidative Stress in the Brain of Pups

The results of lipid peroxidation (LPO) (MDA levels; µmol/mg protein) and antioxidant capacity (TAC µmol/mg protein) tests are displayed in Fig. 3. The MDA measurements in the hypothyroid pups have established a gradual increase

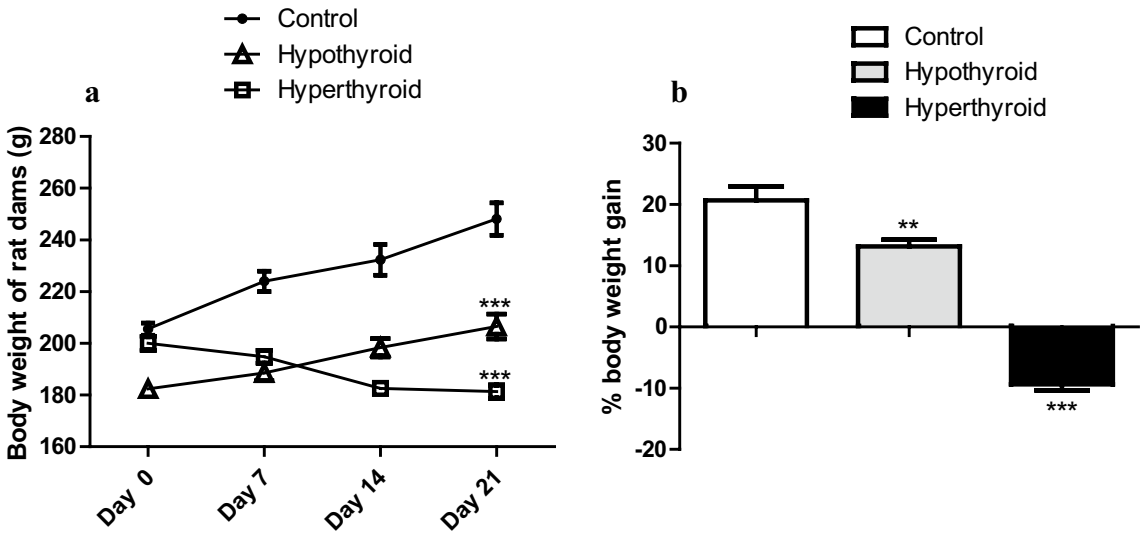

Fig. 2 Analysis of body weight in rat dams. **a** Body weight measurements (Mean ± SEM, n = 8) in day 0 (pre-treatment body weight), day 7, 14, and 21 (1, 2, and 3 weeks after PTU or levothyroxine treatments). **b** Body weight gain percentage. Hypo and hyperthyroid groups were compared versus control group by one way ANOVA. Significance of difference: **p < 0.01 and ***p < 0.001

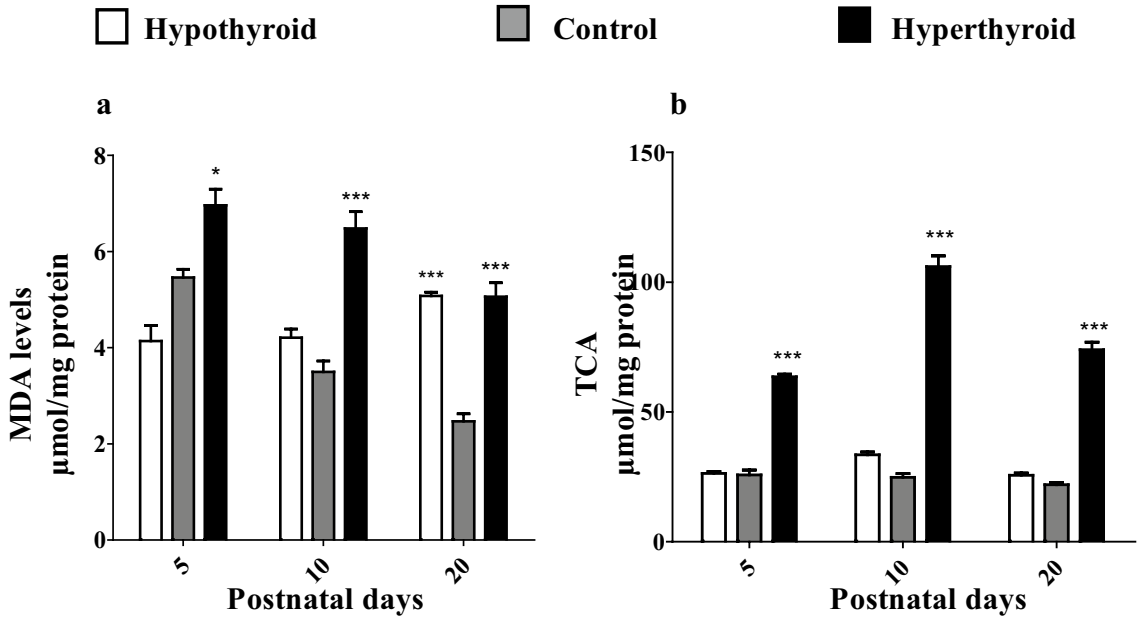

Fig. 3 Effect of maternal hypo and hyperthyroidism on the MDA concentration (µmol/mg protein) (**a**) and TAC (µmol/mg protein) (**b**) in the brain of pups. Values are expressed as mean ± SEM, n = 5. Hypo and hyperthyroid groups were compared versus control group. Significance of difference: *p < 0.05, **p < 0.01 and ***p < 0.001

with age. This rise was meaningful (Tukey's test; $p < 0.001$) only in the brain of PND20 pups as compared to the levels in the age-matched controls (Fig. 3a). However, there were no significant differences in the levels of TAC in the brain of pups in different postnatal days with the control group ($p > 0.05$; Fig. 3b). Both MDA ($p < 0.001$; Fig. 3a) and TAC ($p < 0.001$; Fig. 3b) levels in the hyperthyroid pups on PND5, PND10, and PND20 increased compared to those of the control group.

Apod Level in the Brain of Offspring

The *Apod* mRNA level in the total brain homogenate of pups was studied by the qRT-PCR assay. We showed that

maternal hyperthyroidism significantly increased the *Apod* mRNA level in the brain of hyperthyroid pups on PND5, PND10, and PND20 compared to the expression level in control pups in three age groups (Fig. 4). We found that maternal hypothyroidism significantly increased the *Apod* mRNA level in the brain of pups on PND5 and PND10; however, this increase in the 20-day old hypothyroid group was not significant (Fig. 4).

APOD$^+$ Cells in Different Hippocampal Regions of the Offspring

We analyzed the number of APOD$^+$ cells in the *dentate gyrus* (DG), CA1, CA2, and CA3 hippocampal regions of hypothyroid, hyperthyroid, and control pups on PND5, PND10, and PND20 by the immunohistochemical technique (Fig. 6). We count APOD$^+$ cells in five sections per animal and five fields per sections by Motic Images plus 2.0. As shown in Fig. 5, the analysis of APOD staining indicated that both maternal hypo- and hyperthyroidism significantly increased the number of APOD$^+$ cells in the DG (Fig. 5a), CA1 (Fig. 5b), CA2 (Fig. 5c), and CA3 (Fig. 5d) hippocampal regions of the pups' brain on PND5, PND10, and PND20 in comparison with the control group. Counting the number of neurons in particular regions of the hippocampus stained with H & E determined that the number of neurons decreased significantly in the hypothyroid and hyperthyroid groups compared to the control group. This decrease was higher in the hypothyroid group in the DG, CA1, and CA3 regions (Fig. 7).

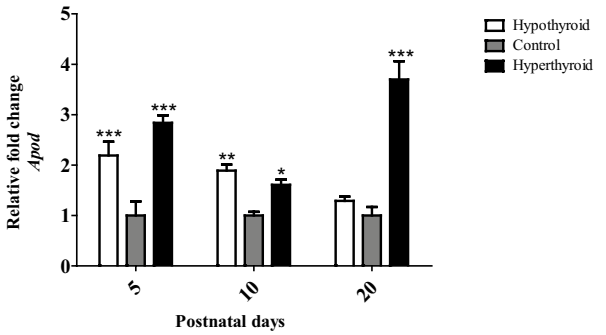

Fig. 4 Effect of maternal hypo and hyperthyroidism on the *Apod* mRNA level (fold change) in the brain of the pups at PND5, PND10. Values are expressed as mean ± SEM, n = 5. Hypo and hyperthyroid groups were compared versus control group. Significance of difference: (ANOVA; LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001)

Discussion

One of the proposed functions of APOD in the brain is oxidative stress protection. It has been highlighted as a brain antioxidant in previous studies [21–23, 34]. THs disorders have profound effects on the genes expression, brain development, and oxidative stress in the central nervous system. We showed in hypo- and hyperthyroid rat pups, APOD expression in hippocampus regions increases. We confirmed the up-regulation of the APOD gene in the pup's hippocampus by immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (qRT-PCR).

There is a scarcity of studies on the effects of thyroid disorders and resultant oxidative stress on *Apod* gene expression. Just two study performed genomic analysis on gene expression profile in the neocortex and hippocampus of hypothyroid rats [35, 36]. In these studies, various genes were evaluated by Affymetrix GeneChip hybridization microarray. *Apod* was one gene has been reported that slightly down-regulated. Our results contradicted the results of Royland et al. and Shiraki et al.

In the Rayland et al. study, the effects of 10 (in the first phase), 1, 2 and 3 ppm PTU, investigated by microarray gene profiling in the hippocampus. It has been reported a lack of direct gene correspondence for microarray results. They mentioned several causes including; variety in the material used for microarray, the brain region specimen's limitation, heterogeneous expression of the genes in a brain region, and one time snapshot for global expression assay (PND14). They believed that changing the gene expression profile for evolution in the brain does not require severe hypothyroidism and many genes were affected by low doses (1 ppm) of PTU in dams and pups. A false discovery rate error of 5% is also reported in this study. *Apod* was one gene that reported down-regulated (up to two fold) as dose-dependent manner at 1, 2, and 3 ppm PTU. They have presented no details of variations in *Apod* gene expression in 10 ppm PTU [35]. In Shiraki et al. investigation, they compared the effect of mild and severe hypothyroidism on global gene expression of DG. However, the *Apod* expression showed a slight increase at a dose of 0.1 mg/kg body weight but decreased by two fold in of 10 mg/kg body weight PTU in the adult's male rats [36].

In microarray methods the fold change with a cutoff of 1.8 to 3 is used for the global gene expression evaluation. In these cases, an inherent error for using the fold change is undeniable. With genes whose absolute expression is low, the probability of this inherent error is higher. So, necessary to confirm the microarray results by qRT-PCR [37]. For the costly nature of the microarray method and low replicates, calculation of the exact variation of the

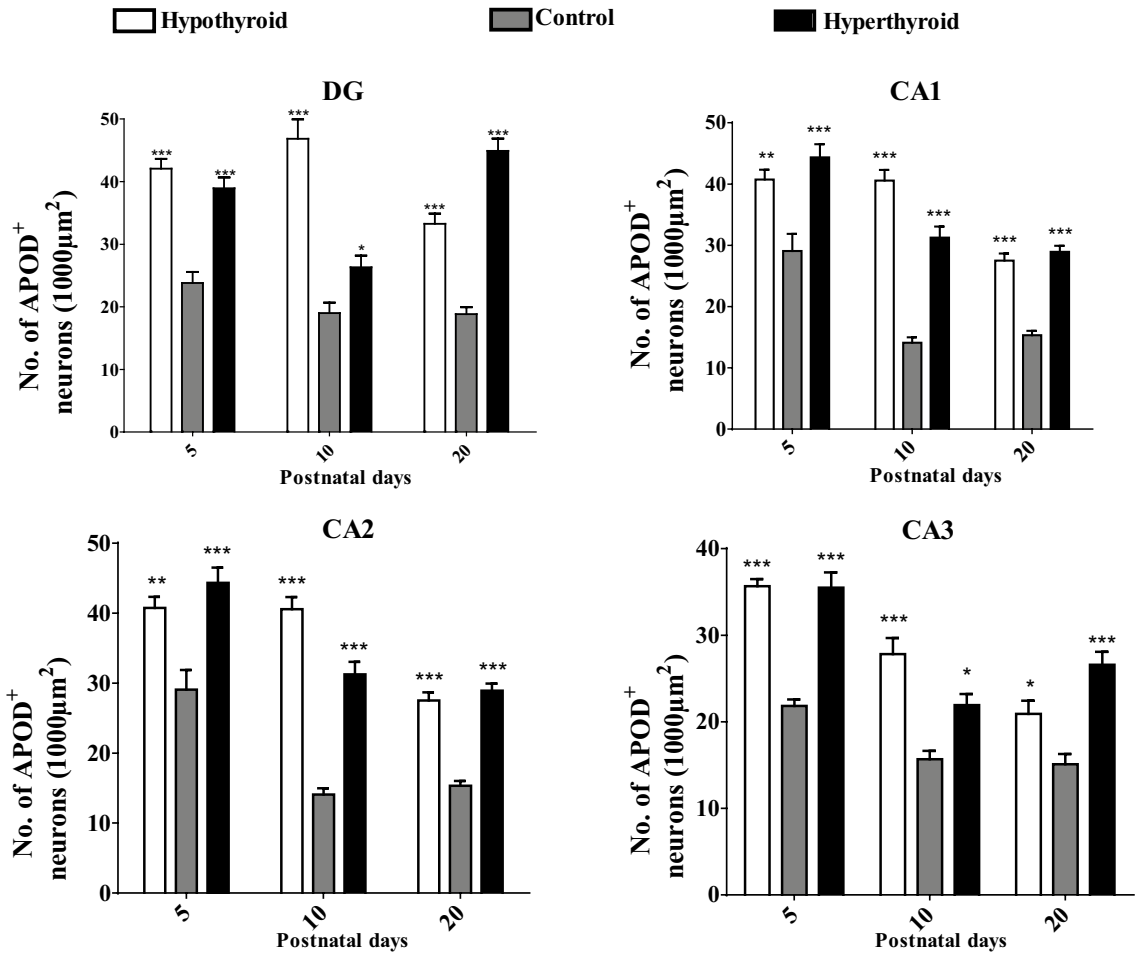

Fig. 5 Effect of maternal hypo and hyperthyroidism on the number of $APOD^+$ neurons in hippocampal regions of pups on PND5, PND10 and PND20. The estimate of $APOD^+$ neurons were determined by the motic image plus 2 software (20×; 1500 pixel; 1000 square micrometer, sq µm). Values are expressed as mean ± SEM. Hypo and hyperthyroid groups were compared versus control group. Significance of difference: (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

changes is difficult. So, in this method where the expression of genes is very low, the results are not necessarily confirmed by qRT-PCR [37]. In mentioned works qRT-PCR didn't carry out for *Apod* expression pattern confirmation [35, 36]. We studied changes in gene expression in 3 evolutionary stages, and IHC results in these three phases (PND5, PND10, and PND20) were consistent with the qRT-PCR results.

Based on the work of Royland et al. research, *Apod* expression was reported in hippocampus of pups in mild hypothyroidism, while in our investigation, we applied 100 ppm PTU for severe hypothyroidism induction. Regarding the results of the Shiraki et al., it seems unlikely that the only PTU concentration causes the discrepancy. According to the Mutch et al. study, it may relate to the technique adopted for the *Apod* expression experiment in other investigations. We didn't find any study in which they measured *Apod* expression in the hippocampus by specific primer or antibody in thyroid disorders.

Rahaman et al. showed that overt hypothyroidism within the first month of pups brain development might lead to increased oxidative stress and lipid peroxidation (LPO) [38]. It has been reported that the levels of LPO and reactive oxygen species increased in the hippocampus and amygdala regions of the rat brain after three weeks of treatment with methimazole. In addition, there was selective oxidative stress in the hypothyroid rats' hippocampus and amygdala where eNOS was involved [39]. These studies confirm our study findings (Fig. 3). Also, administration of levothyroxine (100 µg/kg) to the rabbit for 21 days can cause LPO. They associate several signs and symptoms of hyperthyroidism with oxidative stress [40–43]. Studies by Mogulkoc et al. in hypothyroid rats showed that administration of a high dose of T4 resulted in an increase in oxidative damage in the brain

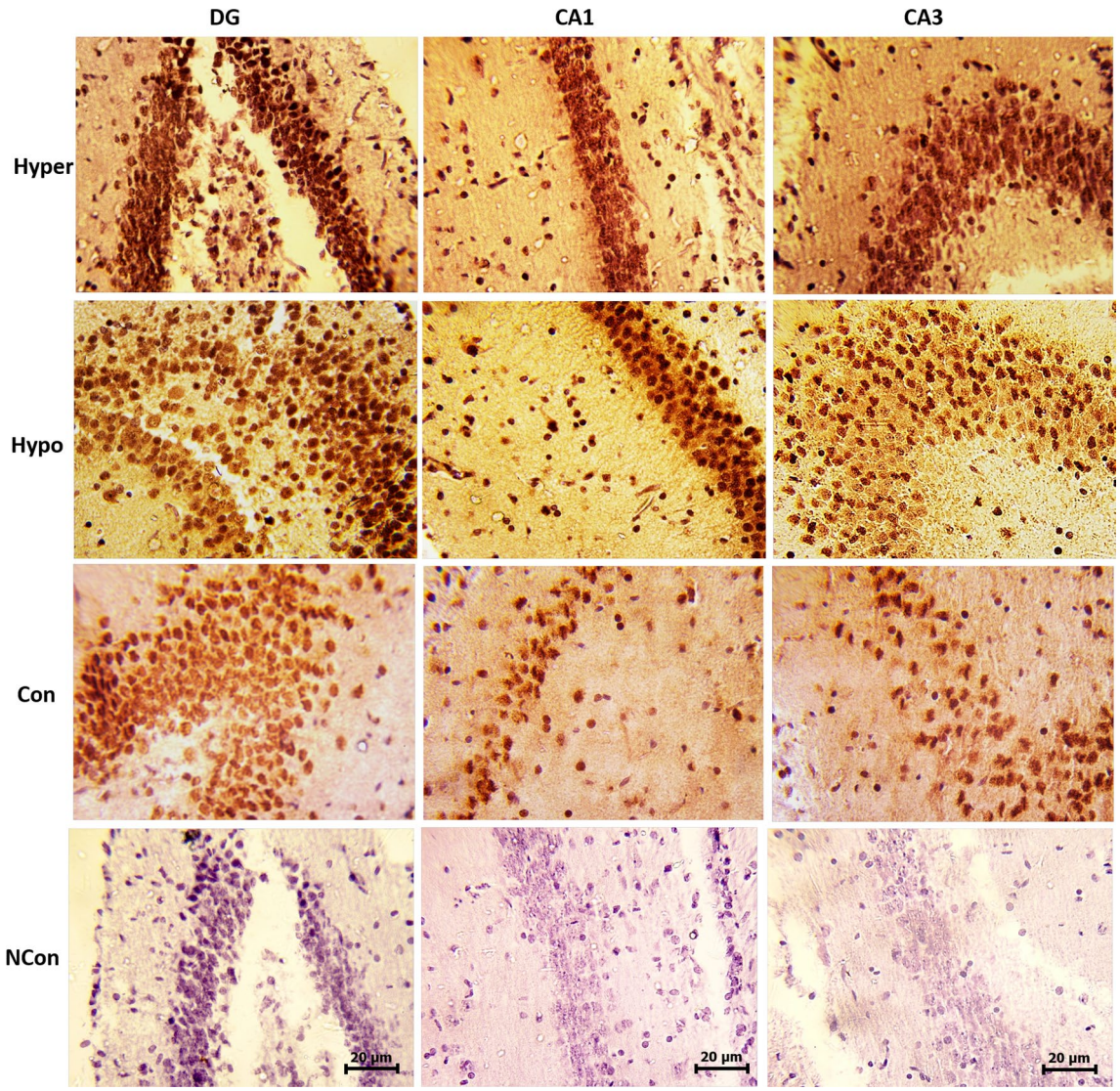

Fig. 6 APOD positive neurons ($APOD^{+}$) in hippocampal regions of pups at PND20 (*Con* Control group, *NCon* Negative Control, *Hypo* Hypothyroid group, *Hyper* Hyperthyroid group

and, although the antioxidant activity also increased, cannot prevent this injury [44].

It was demonstrated that during the development of the nervous system, APOD is secreted by the astroglial cells and increases the survival of the cells [45]. Also, in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, the aging of APOD expression increases. The previous studies proposed that APOD is part of a natural defense against the stress caused by oxidation and inflammation. Given that the promoter of APOD gene has different potential response elements, individual factors associated with these responses can alter its expression, including acute phases, oxidative stress, thyroid hormones, and inflammatory conditions [46]. The presence of three residues of methionine in this protein suggests that this protein has an antioxidant role. Kim et al. study showed that the increase of *Apod* expression is proportional to the increase of superoxide dismutase (SOD) and glutathione peroxidase (GPx) expression in the human prefrontal cortex [47]. In our study, in the hyperthyroid group, on the PND5, PND10, and PND20, the antioxidant capacity increased as compared to the control group of that day. MDA levels as a marker of LPO measured on PND5, PND10, and PND20 were significantly higher in the hyperthyroid group than in the control group. There was coordination between *Apod* expression and LPO in the hyperthyroid group (Figs. 3–5). Based on our results, *Apod* expression in the hippocampus of rat pups in hyperthyroid groups increased, proportional to the biochemical changes of oxidative stress caused by hyperthyroidism.

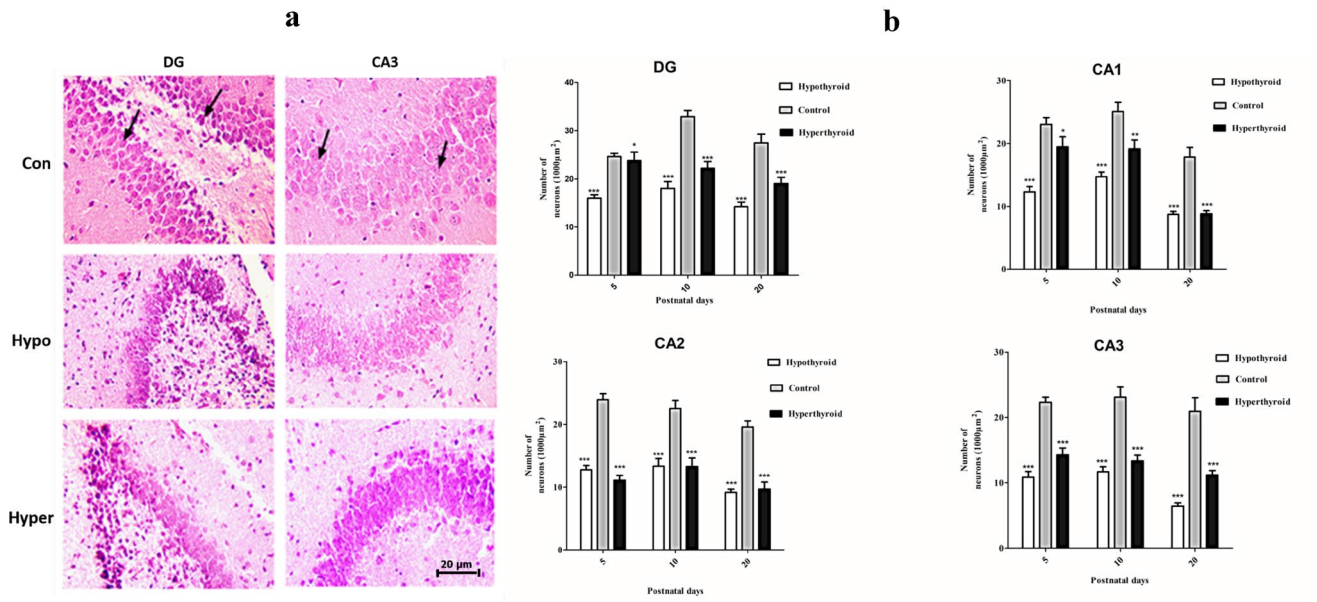

Fig. 7 **a** H & E staining of hippocampal regions sections. **b** Neurons counts in hippocampal regions of pups at PND20 (*Con* Control group, *Hypo* Hypothyroid group, *Hyper* Hyperthyroid group). The estimate of neurons number were determined by the motic image plus 2 software (20×; 1500 pixel; 1000 square micrometer, sq μm). Values are expressed as mean ± SEM. Hypo and hyperthyroid groups were compared versus control group. Significance of difference: (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

In the hypothyroid group, LPO increased significantly only on PND20. The results of our investigation are almost in line with the study by Rahaman et al. The MDA level in the brain of 5, and 15 day old pups did not differ with the control group, but its increase in the brain of 25 day old pups was significant [38]. In the hypothyroid group, the expression at the mRNA level was not directly correlated with LPO. In other words, on PND5 and PND10, LPO did not change in spite of a significant increase in the expression of *Apod*. Regarding the results of IHC in the hypothyroid group, $APOD^+$ cells increased significantly compared to the control group on different days (Figs. 5 and 6). Therefore, according to the results, it seems that oxidative stress is not the only effective factor in regulating APOD gene expression in hypothyroid rats and other factors may also be involved. There is some evidence that, besides the oxidative stress situation, the mediators of inflammation, cell proliferation, and APOE as a regulatory protein also play a role.

Najyb et al. studied the effects of kainic acid induced lesion on transgenic rat's over-expressed human *APOD*. In the exposure with kainate were observed a milder seizure and less pro-inflammatory responses and apoptosis in the hippocampal neurons. It was also found that the high expression of APOD in these rats altered lipid metabolism and moderated the cholesterol content in the neurons [48]. Desmarais et al. showed that high APOD expression in fat tissue of obese women is associated with lower levels of pro-inflammatory factors such as tumor necrosis factor alpha (TNF-α) in the blood [49]. One role of APOD in neurodegenerative diseases is the arachidonic acid stability in the membrane. So, APOD prevents the arachidonic acid release and inflammatory mediator's production [12]. There is evidence that hypothyroidism is associated with cognitive impairments in humans and rats [8, 13–16]. Regarding, hippocampal inflammation association with cognitive deficits [17], hypothyroidism may be associated with increased concentrations of COX-2 and pro-inflammatory mediators [18]. Understanding the assumed mechanism for increasing APOD expression by inflammation in the hypothyroidism is very important. Perhaps measuring APOD levels in CSF is effective in estimating the severity of inflammation and cognitive impairment in thyroid disorder and CNS problems. The study of Nam et al. on the hypothyroid hippocampus showed a high expression of cyclooxygenase-2 (COX-2) and pro-inflammatory factors, while the cell proliferation marker, Ki67, was reduced [50].

Although in our study, the expression of *Apod* mRNA and protein levels in the hypothyroid group was not related to MDA levels on the PND5, PND10, and PND20, but could be related to a significant decrease in cell proliferation in different areas of the hippocampus. We studied the implement of hypo and hyperthyroidism on the number of neurons in various areas of the brain hippocampus in PND5, PND10, and PND20 (Fig. 7). Data analysis showed a considerable reduction in the number of nouns in both groups, in the hypothyroid group. Therefore, it is suggested the growth arrest-induced *Apod* gene expression determinants and COX-2 be tested along with APOD in future works.

Some regulatory proteins such as APOE can be bound to the Apod gene promoter. APOE is an important apolipoprotein in neurodegenerative diseases that shows an inhibitory effect on *Apod* gene expression. An inverse relationship has been observed between the expression of APOD and APOE in the hippocampus of mice [51]. On the other hand, it has been observed that THs modulate *Apoe* gene expression in the brain [52]. In the hypothyroidism group, despite the low LPO (on PND5 and PND10), the high level of *Apod* expression may be associated with a decrease in *Apoe* expression, alleviating its inhibitory effect on the *Apod* promoter.

In addition to the direct and indirect antioxidant role of APOD in the external oxidative stress caused by paraquat, this protein performs other activities, including the transfer of arachidonic acid, progesterone, and retinoic acid to the cells [23]. Increased APOD expression in thyroid disorders in our study may be due to the need for the transfer of compounds such as arachidonic acid and retinoic acid by APOD. A study has shown that a high-fat diet can have differential effects on the expression of *Apod* gene in male rats' sexual organs [53]. Therefore, it is possible that the metabolic changes induced by THs disorders cause the up regulation of *Apod* gene expression in our study.

In sum, our observation for the first time indicated APOD up regulated in severe hypo- and hyperthyroid disorders in the hippocampus and DG of rats. Although, the APOD expression in the hypothyroid group was not related to MDA levels but could be associated with a reduction in cell proliferation in different zones of the hippocampus. Considering the results of our investigation and other studies up regulation of APOD in the hippocampus of hypothyroid pups arises from the carry out of some factors such as reducing cell proliferation, increasing pro-inflammatory mediators, the increase in oxidative stress, and extensive metabolic changes. We suggest a simultaneous assessment of all these issues in future works.

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Compliance with Ethical Standards

Conflict of interest We declare that there are no conflicts of interests exist.

Ethical Approval The ethics committee of Semnan university of medical sciences accredited the study (approval ID: IR. SEMUMS. REC.1395. 215). All experiments and animal care performed based on approved international and national guidelines.

Research Involving Human and Animal Participants Authors have performed no studies on the human participants in this study.

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