

S100 Expression in Astrocytes of the Developing Cerebella of the Offspring Rats of Hyperthyroidism Mothers (Biochemical, Histological and Immunohistochemical Studies)

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ABSTRACT

Background: Thyroid dysfunctions are the second most common endocrinological disorders in pregnancy after diabetes mellitus. Uncontrolled and inadequately treated maternal hyperthyroidism may result in fetal and neonatal hyperthyroidism that may affect the cerebellar development. Astrocytes are target cells for thyroid hormones. **Aim:** To verify the effect of maternal thyrotoxicosis on the biochemical and histological architecture of the offspring's developing cerebellum. **Material and Methods:** Thirty pregnant rats were divided into two equal groups control and hyperthyroidism. The cerebella of all offspring of both the groups were examined in 1st, 3rd, 5th and 7th postnatal days. One hemisphere was used for histological and immunohistochemical study and the other for biochemical investigation. Also, estimation of Thyroid Hormone levels and Glutathione Peroxidase in the serum was done. **Results:** The offspring of the treated rats showed a significant increase in the levels of T3, and T4 in all studied ages ($p < 0.001$). In addition, the homogenized fluid of the cerebella from all offspring of treated rats showed decreased levels of Glutathione Peroxidase levels and increased Malondialdehyde levels ($p < 0.001$ for both) indicating high oxidative stress and low in antioxidant defense. Light microscopic investigation of the cerebellar cortex of the offspring of the treated rats showed marked growth retardation in the cerebella in the form of shallow fissures and delayed newly formed ones. Some degenerative changes were observed in the four layers of the cerebellar cortex; some cells have vacuolated cytoplasm with the presence of interstitial hemorrhage during all studied ages. The dendritic arborization of Purkinje cells of experimental animals showed degeneration in the form of less branching dendrites or fuse with adjacent ones. Application of S100 immunostaining revealed that the most affected and expressed cells were the astrocytes which appear markedly immunoexpressed and altered morphologically showing enlarged irregular cells with multiple thick disrupted processes. **Conclusion:** Hyperthyroidism in rats during gestation and lactation periods badly affected the development of cerebellum of the offspring at all examined period. Significantly, astrocytes altered morphologically. The disturbed cerebellar cytoarchitecture might be due to increase oxidative stress and impaired antioxidant defense system.

Keywords: Induced Maternal Hyperthyroidism, Oxidative Stress, Offspring Developing Cerebellum, Astrocytes, S100.

INTRODUCTION

Thyroid disorders are the second most common endocrinological disorders in pregnancy after diabetes mellitus (Abalovich et al., 2007). Thyroid hormones are critical for the development of the fetal and neonatal brain. These hormones were contributed on neural and glial lineages and control cell proliferation, apoptosis, migration, and differentiation (Baas et al., 2002). These actions are most apparent in central nervous system development (Shwartz, 1983). Thyroid hormones appear to have their most intense effects on the terminal stages of brain differentiation, including synaptogenesis, growth of dendrites and axons, myelination and neuronal migration (Oppenheimer and Schwartz, 1997). The results of Sevilla et al. (2002) also showed that thyroid hormone regulates rat neuro-retinogenesis.

Human fetuses acquired the ability to synthesize thyroid hormones at the 12th weeks of gestation. Relevant evidence from several species indicates that there is a trans-placental transfer of the thyroid hormones from the mothers to their offspring. Moreover, the placenta contains deiodinases that can convert T₄ to T₃ (Xue et al., 1994). Uncontrolled and inadequately treated maternal hyperthyroidism may result in fetal and neonatal hyperthyroidism (Zimmerman, 1999) due to the transplacental transfer of stimulatory Thyroid Stimulating Hormone (TSH) receptor antibodies (Polak et al., 2004).

The cerebellum has an outstanding structure consisted of folia divided by fissures of varied in depth. The development of cerebellar foliation originated before birth and its adult configuration of fissures and folia is achieved postnatal (Bouet et al., 2005). It is composed of few cell types, all ordered in a precise manner in various morphological layers; external granular layer, molecular layer, Purkinje cell layer and internal granular layer (Llinas, 1975).

In rats, during postnatal development of the cerebellum, the external granular cell layer represented the matrix area in which two distinct zones can be visibly recognized, the proliferative and the premigratory zones (Altman, 1972a-c and Rakic, 1971). The authors suggested that the post-mitotic cells of external granular layer migrated to their final destination in the internal granule cell layer. Purkinje cells are the single output of the cerebellar cortex (Altman, 1969; 1972a, b and c). There are many stages in the development of Purkinje cells as the cell differentiates from round neuroblast to an adult neuron. Altman (1982) recommended that in rats, the Purkinje cells variation occur during late embryonic and early postnatal periods. Sonic hedgehog factor secreted by the Purkinje cells regulated the number of folia through its influence on granule cell precursor proliferation (Corrales et al., 2004 and 2006). The majority of granule cells had practiced some substantial developmental milestones postnatal. Sotelo (2004) explained that the proliferating granule cells precursors are prenatally positioned in the external granular layer followed by inward radial migration to their final destination in the internal granular layer.

Astrocytes make up 20 to 50% of the volume of most brain areas and they represent a various class of cells that have numerous different roles. They had a vital role in normal brain function and disease (Trentin, 2006). Rowitch and Kriegstein (2010) reported that the astrocytes are macroglial cells in the central nervous system obtained from heterogeneous populations of progenitor cells in the neuroepithelium of the developing central nervous system. The authors added that there is a remarkable similarity between the well-known genetic mechanisms that specify the lineage of diverse neuron subtypes and that of macroglial cells. Astrocytes have

numerous supporting functions such as guiding neuronal migration, maintaining the microenvironment of neurons and preservation of the blood-brain barrier (Janzer and Raff, 1987). They are implicated in synapse formation and function (Rossi, 2015). Astrocytes supported immune defense by producing various immunoreactive cytokines (Benveniste, 1992). Recent investigations have emphasized the vital role of astrocytes in various homeostatic functions within the central nervous system and their role in bi-directional communication with neurons by releasing neuroactive substances. They play an active role in the secretion of substances regulating metabolism and substances regulating synaptic transmission, including neurotransmitters and neuromodulators (Petrelli and Bezzi, 2016). The character of astrocytes can alter in parallel with, or as a consequence of the morphological, biochemical and functional changes undergo upon injury or disease. As a result, they have the possible to convert from helpful supports and cooperating partners for neurons into harmful enemies (Rossi, 2015).

The S100 protein belongs to the EF (helix E-loop-helix F) hand family of calcium binding proteins (Donato, 1991). The name "S100" was given because of their solubility in a 100% saturated solution with ammonium sulfate. At least 25 proteins have been identified as belonging to the S100 protein family (Marenholz et al., 2004). S100B was principally produced by astrocytes in the central nervous system convoluted in several pathologies and represented astrocytic activation (Steinacker et al., 2013).

S100B was situated in the cytoplasm and nucleus of the astrocytes along with other members of the S100 family. Moreover, S100B has regulated the cytoskeletal structure and cell proliferation (Gonçalves, 2008 and Yarden et al., 2011). Steiner et al. (2007) reported that the astrocytes are the predominant S100B-positive cells in the gray matter while the oligodendrocytes are the predominant S100B-positive cells in the white matter.

Several pieces of investigations supported that the neurons are protected against the reactive oxygen species (ROS) induced toxicity of various compounds and drugs in the presence of astroglial cells. These observations confirmed by Desagher et al. (1996) and Dringen et al. (2000). As they induce the transcriptional up-regulation of the glutathione synthesis (Iwata-Ichika et al., 1999; Dasgupta et al., 2007). It is well-known that during brain development, astrocytes are target cells for thyroid hormones (Clos and Legrand, 1973; Legrand et al., 1976 and Gould et al., 1990).

Based on the previous data, the current study was prompted to verify the effect of maternal thyrotoxicosis in rats on the biochemical and histological architecture of its offspring developing cerebellum.

MATERIAL AND METHODS

Animals

The current research was carried out on 48 adult fertile albino rats, 32 females, and 16 males, weighing about 165–190 gm. The rats were housed in Animal Care Center of Faculty of Medicine for Girls, AL-Azhar University. The rats were reserved under observation for two weeks to exclude any contamination and to acclimatize the new surroundings. The selected rats were housed in stainless steel cages at a normal atmospheric temperature (24 ± two °C) and fed on standard rodent pellet. Diet manufactured by the Egyptian Company for

Oil and Soap (Cairo, Egypt). Tap water was used for drinking ad libitum, and these animals were maintained at constant daily light/dark periods of 12 hours each.

Experimental Animals

Mating was induced by housing two females with one male in a separate cage overnight for one or two successive days. Thirty female rats have the mucous plug; the vaginal smear was found to contain cornified non-nucleated epithelial cells, leukocytes, and a large quantity of mucous (Paull and Fairbrother, 1985). The pregnant females were moved into separate cages from males to start the experiment. The experiments were conducted according to the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

Experimental Schedule and Protocols of Induction of Maternal Thyrotoxicosis

The pregnant rats from the first day of pregnancy, gestation day 1 to lactation day 7 (GD1-LD7) were distributed into two equal groups as follow:

Group I, Control group: The pregnant rats received distilled water via gavage tube daily from the first day of gestation up to the seventh day post-partum.

Group II, Hyperthyroidism group: The pregnant rats received 50 µg/kg body weights /day of Eltroxin produced by GlaxoSmithKline, Cairo, A.R.E. dissolved in distilled water at 9:00 am according to Ahmed (2006) from gestation day one to lactation day seven. The cerebella of all offspring of both the control and treated groups in 1st, 3rd, 5th and 7th days postnatal were examined. One cerebellar hemisphere was selected for histological study and the other for biochemical investigation.

BIOCHEMICAL ANALYSIS

Estimation of Thyroid Hormone Levels

Serum thyroid hormones (T3 and T4) levels were investigated in pregnant rats and all their offspring at the day 1 to confirm their exposure to hyperthyroidism. Blood samples were collected through the retro-ocular puncture. After that, blood was centrifuged (3000 rpm for 20 min) and sera were separated and stored at -20 Co.

RIA for Total TSH, T4 and T3

Serum total T4 and total T3 levels were determined with Amerlex RIA kits (Amersham International, Buckinghamshire, UK), according to the manufacturer's instructions (Sewall et al., 1995). Serum TSH levels were determined with a rat's TSH enzyme immunoassay kit (Amersham International).

Estimation of Oxidative Stress Markers

Measurement of Anti-Oxidant Activity; Glutathione Peroxidase (GPx):

To estimate Glutathione peroxidase (GPx), the samples were homogenized in 1.15% KCL solution. Glutathione peroxidase (GPx) level in cerebellar tissue was estimated by the method described by Paglia and Valentine (1967). The GPx enzyme activity is represented as Unit/mg protein.

Measurement of Oxidative Stress marker: Tissues Malondialdehyde (MDA):

Tissues Malondialdehyde was determined by the method of Mitsuru and Midori (1978). The amounts of lipid peroxides calculated as nMol/ml.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS; version 18.0) was used for analysis of the biochemical data. The unpaired t-test is used to compare the data of each variable with that of the control. All data were represented as mean ± standard deviation (SD). P < 0.05 was considered to be statistically significant.

Histological Preparation for Light Microscope Investigation

Midsagittal sections of brains of all offspring of the control and the treated rats were dissected and processed for histological study. They were fixed in 10% neutral formalin for 48 h. Tissues were dehydrated in ascending concentrations of alcohol, cleared in xylene, and embedded in paraffin. Five-micrometer-thick sections were prepared and stained with Haematoxylin and Eosin for histological examination according to the method of (Bancroft and Gamble, 2013).

Immunohistochemical staining for Detection of S100 Expression

Formalin-fixed cerebellum sections were deparaffinized and antigen demasking was performed by using 20 minutes heat-induced epitope retrieval in Dako Target Retrieval Solution. Pre-incubation with 1.5% H₂O₂ for 10 min to block endogenous peroxidase activity was followed by blocking of nonspecific binding sites with 10% normal goat serum for 60 min and repeated washings with PBS. The primary antibody was diluted in PBS and used at a dilution of 1:400 and applied for 48 h at 4°C. Antibodies were from (DAKO, Glostrup, Denmark: Polyclonal Rabbit Anti-S100). The immunostaining was amplified and completed by Horseradish Peroxidase complex (Dako EnVision™+/HRP kits).

An undiluted HRP-conjugated polyclonal was used as the secondary antibody. Sections were developed and visualized using 3,3-diaminobenzidine (Dako REAL™ DAB+ Chromogen) at room temperature. The substrate system produces a crisp brown end product at the site of the target antigen. Cells labeled by the antibody displayed staining confined to the cytoplasm. Sections were counterstained with Mayer's haematoxylin. The sections were then dehydrated in alcohol, cleared in xylene and coverslipped with Permount. Applying immunohistochemical methods to the tissues showed that the bulk of S100 has been localized in cell bodies, processes and lamellae of astrocytes as a brown colour (Michael and Joachim, 1995).

RESULTS

The Results of Biochemical Analysis

The pregnant rats treated with Eltroxin showed a significant increase in the levels of T3 (10.46±0.78) and T4 (16.02±0.85) and a significant decrease in the level of TSH (0.96±0.09) when compared with their corresponding levels in the pregnant control (6.14±0.19; 11.95±0.46; 1.67±0.14) P<0.001 (Table 1).

Table 1: Comparison of biochemical analysis in pregnant rats treated with Eltroxin with controls

Biochemical analysis	Pregnant rats treated with Eltroxin Mean (\pm SD)	Pregnant controls Mean (\pm SD)	p-value
T3	10.46 (\pm 0.78)**	6.14 (\pm 0.19)	p<0.001
T4	16.02 (\pm 0.85)**	11.95 (\pm 0.46)	p<0.001
TSH	0.96 (\pm 0.09)**	1.67 9(\pm 0.14)	p<0.001

SD-Standard deviation TSH-Thyroid Stimulating Hormone

** p-value less than 0.001

Table 2: Comparison of biochemical analysis on offspring of Eltroxin treated rats with offspring of controls

Biochemical Analysis	offspring From Eltroxin Treated Rats Mean (\pm SD)	Biochemical Analysis	offspring From Control Rats Mean (\pm SD)
T3		T3	
TPD1	9.60 (\pm 0.82)**	CPD1	6.08 (\pm 0.22)
TPD3	10.06 (\pm 0.06)**	CPD3	6.14 (\pm 0.22)
TPD5	10.14 (\pm 0.58)**	CPD5	6.06 (\pm 0.05)
TPD7	10.18 (\pm 0.19)**	CPD7	6.22 (\pm 0.10)
T4		T4	
TPD1	16.44 (\pm 0.51)**	CPD1	10.60 (\pm 0.72)
TPD3	16.53 (\pm 0.29)**	CPD3	11.38 (\pm 0.73)
TPD5	16.76 (\pm 0.21)**	CPD5	11.60 (\pm 0.45)
TPD7	16.56 (\pm 0.69)**	CPD7	11.61 (\pm 0.39)
TSH		TSH	
TPD1	1.026 (\pm 0.016)**	CPD1	1.67 (\pm 0.07)
TPD3	1.028 (\pm 0.013)**	CPD3	1.73 (\pm 0.07)
TPD5	1.028 (\pm 0.01)**	CPD5	1.71 (\pm 0.06)
TPD7	1.028 (\pm 0.02)**	CPD7	1.73 (\pm 0.04)

TPD1- Treated postnatal day one; TPD3-Treated postnatal day 3; TPD5-Treated postnatal day;

TPD7- Treated postnatal day 7; CPD1-Control postnatal day 1; CPD3-Control postnatal day 3;

CPD5-Control postnatal day 5; CPD7-Control postnatal day 7.

SD- Standard deviation ** p-value less than 0.001

Table 3: Comparison of antioxidant activity (Glutathione Peroxidase-GPx) and oxidative stress activity (Tissues Malondialdehyde-MDA) of offsprings from Eltroxin treated rats with offsprings from controls.

Biochemical Analysis	Antioxidant Activity In offspring From Eltroxin Treated Rats Mean (\pm SD)	Antioxidant Activity In offspring From Control Rats Mean (\pm SD)
TPD1	1.876 (\pm 0.04)**	2.78 (\pm 0.15)
TPD3	1.835 (\pm 0.14)**	
TPD5	1.79 (\pm .04)**	
TPD7	1.74 (\pm 0.51)**	
	Oxidative Stress Activity In Offspring From Eltroxin Treated Rats Mean (\pm SD)	Oxidative Stress Activity In offspring From Controls Mean (\pm SD)
TPD1	1.050 (\pm 0.03)**	0.57 (\pm 0.04)
TPD3	1.73 (\pm 0.04)**	
TPD5	1.70 (\pm .06)**	
TPD7	1.73 \pm 0.09)**	

TPD1- Treated postnatal day one; TPD3-Treated postnatal day 3;
 TPD5-Treated postnatal day; TPD7- Treated postnatal day 7
 SD- Standard deviation ** p-value less than 0.001

The offspring of the pregnant treated rats showed significant increase in the levels of T3 and T4 at the TPD1, TPD3, TPD5, and TPD7 (T3; 9.60 \pm 0.82, 10.06 \pm 0.06, 10.14 \pm 0.58 and 10.18 \pm 0.19- T4; 16.44 \pm 0.51, 16.53 \pm 0.29, 16.76 \pm 0.21, and 16.56 \pm 0.69) when compared with the corresponding levels of offspring for pregnant control rats at CPD1, CPD3, CPD5 and CPD7 (T3; 6.08 \pm 0.22, 6.14 \pm 0.22, 6.06 \pm 0.05 and 6.22 \pm 0.10- T4; 10.60 \pm 0.72, 11.38 \pm 0.73, 11.60 \pm 0.45 and 11.61 \pm 0.39) P<0.0001 for all. The offspring of the pregnant treated rats showed significant decrease in the levels of TSH at the TPD1, TPD3, TPD5, and TPD7 (1.026 \pm 0.016, 1.028 \pm . 013, 1.028 \pm 0.01and1.028 \pm .02) when compared with the corresponding levels for offspring of pregnant control rats CPD1, CPD3, CPD5 and CPD7 (1.67 \pm .07, 1.73 \pm 0.07, 1.71 \pm 0.06, and 1.73 \pm 0.04) p<0.001for all (Table 2).

The Anti-Oxidant Activity; Glutathione Peroxidase (GPx) levels showed significant decreases in the homogenized fluid of the cerebellum from the offspring of treated rats at the TPD1, TPD3, TPD5, and TPD7 (1.876 \pm 0.04, 1.835 \pm 0.14, 1.79 \pm .04 and 1.74 \pm 0.51 respectively) when compared with the control (2.78 \pm 0.15) p<0.001. No significant differences in the levels of GPx between rats at different days of lactation p> 0.05 (Table 3).

Oxidative Stress Activity; Tissues Malondialdehyde (MDA) levels showed significant increases in the homogenized fluid of the cerebellum from the offspring of treated rats at the TPD1, TPD3, TPD5, and TPD7 (1.050 \pm 0.03, 1.73 \pm 0.04, 1.70 \pm .06 and 1.73 \pm 0.09 respectively) when compared with the control

(0.57 \pm 0.04) p<0.001. No significant differences in the levels of MDA between rats at different days of lactation p> 0.05 (Table 3).

HAEMATOXYLIN AND EOSIN RESULTS

Control Postnatal Day One (CPD1) Rats

Light microscopic examination of the serial mid-sagittal sections of the cerebellar cortex of (CPD1) showed the normal architecture of lobules and fissures (Fig.1: A&B). The cerebellar cortex consisted of the external granular layer, molecular layer, Purkinje cell layer and internal granular layer from outside inward. The external granular layer consisted of a relatively thick sheet of small darkly stained cells which differentiated into superficial or outer closely crowded zone and an inner or deep less packed zones. The molecular layer was poorly developed pale zone with a loose aggregation of cells. Purkinje cells could barely be noticed, it had relatively large cells mixed together among cells of the internal granular layer or as an unclear layer intermingled with the cells of the superficial zone of the internal granular layer. The internal granular layer was relatively thick layer of darkly stained cells of different size and shape and lying just superficial to the white matter (Fig.1: C).

Treated Postnatal Day One (TPD1) Rats

Examination of the cerebellar cortex of (TPD1) rats displayed a slight reduction of the cerebellar size and its fissures which appeared shallow and poorly developed in comparison with the control one (Fig.1: D&E). There was an apparent diminished in the depth of the fissures. Moreover, there was delayed the appearance of other fissures. The cerebellar cortex showed that there was a slightly diffusion of cortical layers with the presence of cavitation and presence of wide spaces between the cells. The external granular layer was thin and poor differentiated in comparison to the control and its cells were small rounded or oval in shape with intensely stained nuclei. The molecular layer appeared thinner than that of the control one also, it could hardly be identified and showed poor differentiation with multiple cavities. Purkinje cell layer could not be identified comparable to the control group. The internal granular layer contained small rounded deeply stained nuclei with the appearance of several cavities. Moreover, its cells were detached from each other and some of them had vacuolated cytoplasm (Fig.1: F).

Control Postnatal Day Three (CPD3) Rats

Examination of the cerebellar cortex of (CPD3) rats showed the progress of development of lobules and fissures, however, the cerebellum was enlarged in size to increase the depth of the fissures in comparison to the previous control age (Fig.2: A& B). The external granular layer was relatively increased in thickness while the molecular layer was slightly increased when compared with the preceding control age (CPD1). Purkinje cell layer was a clearly observed as multilayers than that of the previous control age. The cells were placed in more than one row intermixed with the cells of the outer zone of the internal granular layer. Moreover, Purkinje cells looked bigger, more oval and paler than those of the internal granular cell layer. Apparently the cells were more developed as compared with the previous control age (Fig.2: C& D).

Treated Postnatal Day Three (TPD3) Rats

Examination of the cerebellar cortex of (TPD3) rats showed that the depth of the fissures was markedly decreased as compared with the control rats of the same age (Fig.2: E& F). The external granular layer showed multiple cavities and the molecular layer seemed to be less developed than that of control rats of the equivalent age. Also, Purkinje cell layer looked to be less differentiated cells, it could not be noticed as an ultimate layer, but they could be observed as intermingled and clumped with the cells of the internal granular layer. In addition, the cells of the internal granular layer were disconnected from each other and some of them had vacuolated cytoplasm. In general, less cellularity and cavitation in the four layers of the cerebellar cortex were clearly observed (Fig.2: G& H).

Control Postnatal Day Five (CPD5) Rats

Examination of the cerebellar cortex of (CPD5) rats showed increased in size and the depth of the fissure as compared with the previous control age (Fig.3: A). The external granular layer was increased in thickness than that of the prior control age. Also, the cells were increased in size and were clearly distinguished into an outer zone with closely packed cells and an inner zone with loosely radially arranged cells. Apparently, the molecular layer was increased in thickness. In addition,

Purkinje cell layer was well developed than that of the prior control age. They were arranged in many rows with rounded or oval nuclei and less pale cytoplasm. Also, the internal granular layer also seemed more developed than the previous control age (Fig.3: B&C).

Treated Postnatal Day Five (TPD5) Rats

Examination of the cerebellar cortex of (TPD5) rats displayed distorted cytoarchitecture and relatively shallow depth of the fissure as compared with the identical control age (Fig.3: D). The external granular layer seemed to be more dispersed. Moreover, the molecular layer contained a less cellular population in comparison with those of the control of the same age. Also, there was an obvious focal loss of the molecular layer in some lobules (Fig.3: E&F). Similarly, Purkinje cell layer had less distinguished in comparison with the previous control of the same age. In addition, there was an observable focal loss in some area of Purkinje cell layer. The cells of the internal granular layer were disconnected from each other and some of them had vacuolated cytoplasm. Multiple cavities were clearly noticed within the internal granular layer. Also, there was a consistent focal loss of cells in this layer in some lobules (Fig.3: F).

Control Postnatal Day Seven (CPD7) Rats

Examination of cerebellar cortex of (CPD7) rats clarified that the cerebellar cortex was well developed in the form of an increase in size and markedly increased with the depth of its fissures. In addition to development and differentiation of new fissures was observed (Fig.4: A). The cerebellar cortex showed the presence of clear, well demarcated four cellular layers (Fig.4: B). The external granular layer and the molecular layer were prominently increased in thickness (Fig.4: B&C). Purkinje cell layer was arranged in one single row and Purkinje cells were typically oval or fusiform in shape with their long axes vertical to the surface. The elongated dendrites of Purkinje cells in the molecular layer and dendritic arbors were clearly observed. Most of Purkinje cells were aligned in a regular pattern (Fig.4:C). The internal granular layer was also well developed and lying superficial to white matter and its cells were rounded with rounded vesicular nuclei and pale acidophilic cytoplasm (Fig.4: B&C).

Treated Postnatal Day Seven (TPD7) Rats

Examination of the cerebellar cortex of (TPD7) rats showed that the cerebellar cortex was less developed. It was obviously decreased in size and its fissures were noticeably shallow (Fig.4: D) as compared with the same control age. In addition, the newly fissures which appeared less differentiated than those of the controlled one. The cells of the external granular layer were clumped together and they appeared small in size with deeply stained nuclei. A reduction in the size of the molecular layer was noticed. Purkinje cells were disordered in one row with the pale stained cytoplasm. It displayed a variable degree of focal cell loss (Fig.4: E&F). Apparently, dendritic arbors of Purkinje cells were less developed and immature (Fig.4: E, F&G). Significantly, the internal granular cell layer was distended and it was fragmented with the presence of vacuolations, cavitation, dilated capillaries with the presence of wide spaces between the cell (Fig.4: E, F&G).

Immunohistochemistry Results

Polyclonal Rabbit Anti-S100 was confined to the cytoplasm of the astrocytes. The expression was moderately positive in the control group, but it is markedly expressed in the treated rats through the examined period from the 1st postnatal day until the 7th postnatal day (Figs.5, 6, 7&8). Moderate S100 expression in the astrocytes in the control 1st postnatal day rats was observed. The Normal shape of astrocytes can be clearly detected (Fig.5: A, B&C). The marked expression in the astrocytes of treated rats at 1st postnatal day, the astrocytes seemed small with few thin, long irregular processes (Fig.5: D, E&F). Also, the expression was predominant in the astrocytes of treated rats at 3rd postnatal day (Fig.6: C, D, E& F) as compared to the control (Fig.6: A& B) of the same age. Hypertrophy of astrocytes of treated rats with S100 enriched processes at the 5th postnatal day (Fig.7: C&D) in comparison to the control of the equivalent age (Fig.7: A& B). Persistent apparent expression in the astrocytes of treated rat at 7th postnatal days (Fig.8: C&D). Significantly astrocytes in the treated rats were changed morphologically, showing enlarged irregular cells with increased number of thick interrupted processes as compared to the control of the equivalent age (Fig.8 A& B).

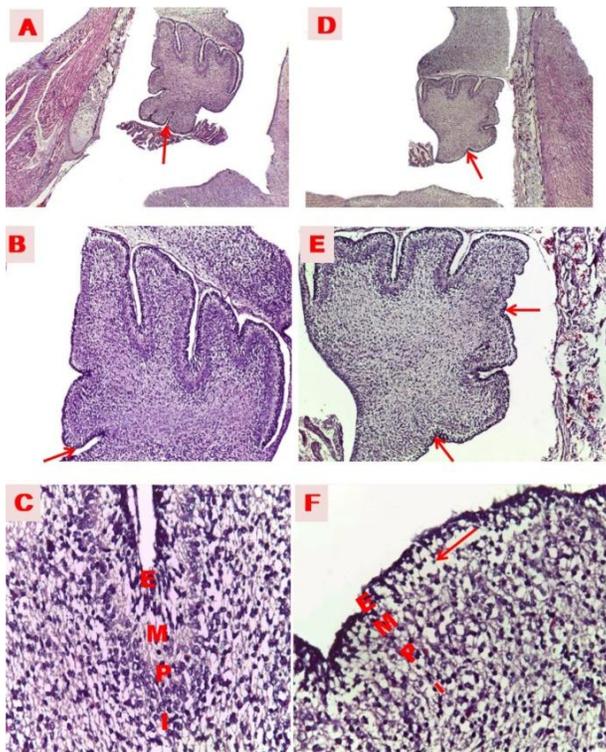


Fig. (1): Photomicrographs of median sagittal sections of the cerebellar cortex of one day old rat (A, B & C are controlled) while (D, E & F are treated) demonstrating:

(A& B): Normal architecture of the cerebellum with normal fissures and lobules (arrows).

(C): The cerebellar cortex consists of four layers; external granular (E) with packed superficial zone and a loose deep zone, molecular layer (M), undifferentiated Purkinje cell layer (P) and the internal granular layer (I).

(D& E): The cerebellum is less developed with marked reduction of the depth of the fissures (arrows).

(F): Poorly developed external granular layer (E). The molecular layer (M) appeared as a very thin narrow zone with less cellularity (arrow). Purkinje cells are not clearly seen. Note, dispersion of the internal granular layer (I) with small size deeply stained cells. (A& D: Hx. & E. x 40); (B& E: Hx. & E. x100) and (C& F: Hx and E. x400).

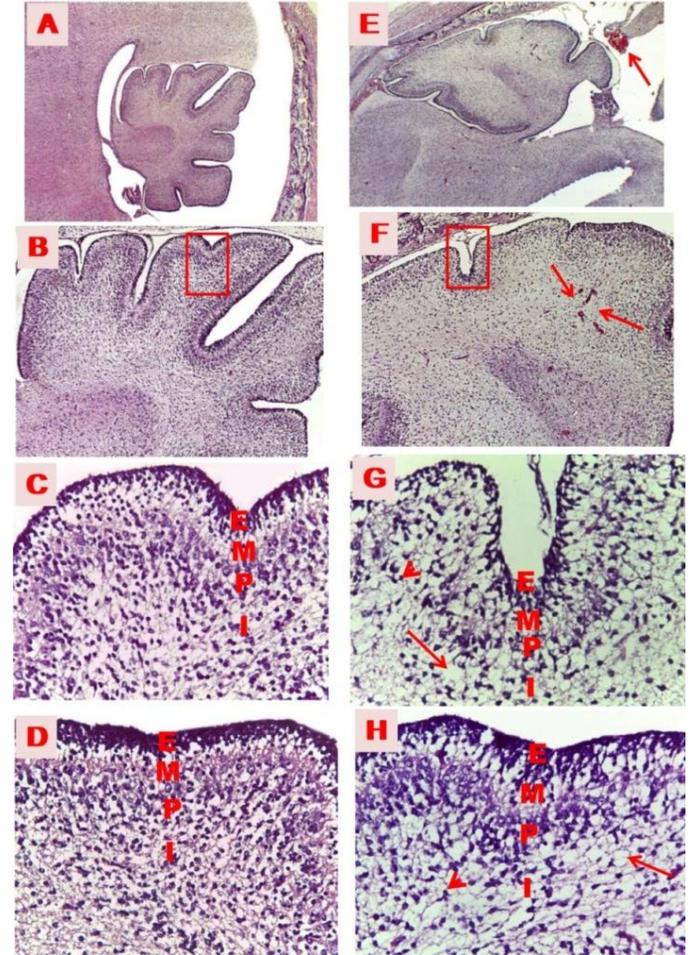


Fig. (2): Photomicrographs of median sagittal sections of cerebellar cortex of three days old rat (A, B, C & D are controlled) while (E, F, G & H are treated) demonstrating:

(A& B): Normal construction and appearance of the cerebellum with normal fissures and lobules.

(C & D): An increase in the thickness of the external granular layer (E) which differentiated into outer closely packed and inner radially arranged zone. Also, an increase in the thickness of the molecular layer (M), Purkinje cells (P) are arranged in more than one row. An increase in the thickness of the internal granular layer (I) is obvious.

(E& F): Apparent decrease in the development of the cerebellum and marked reduction of the depth of the fissures. In (E) Extensive hemorrhage surrounds the cerebellum (arrow) and numerous dilated blood capillaries inside the cerebellum as shown by arrows in (F).

(G&H): Note the layers of cerebellar cortex with less cellularity and some of the cells have pyknotic nuclei (arrowheads). Most of the cells have vacuolated cytoplasm (arrows) in the four layers of the cerebellar cortex.

(A & E :Hx.& E.x40); (B & F :Hx.& E. x100) and(C,D,G& H : Hx&E. x 400).

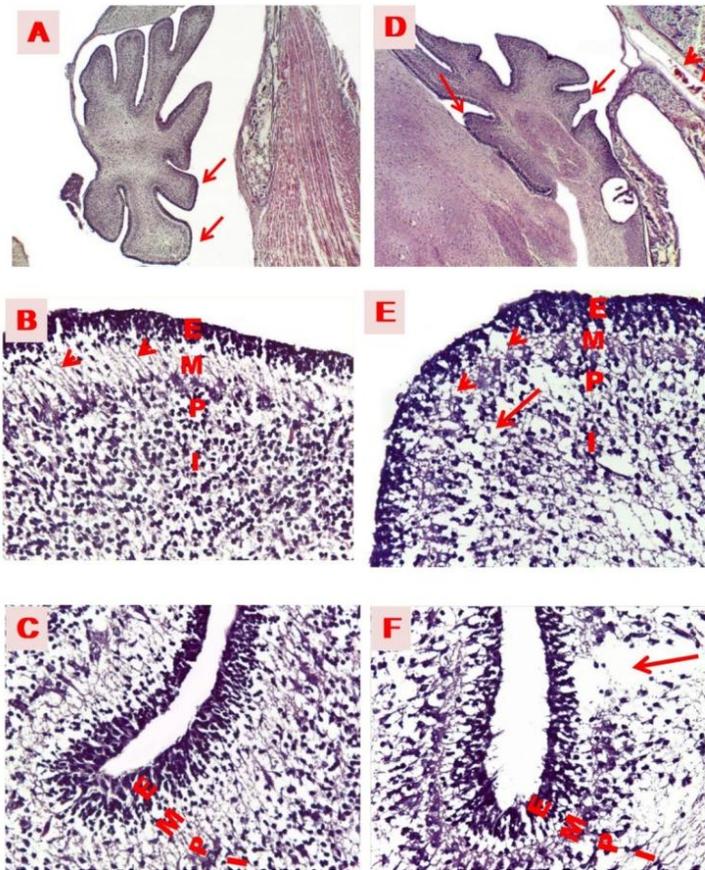


Fig. (3): Photomicrographs of median sagittal sections of the cerebellar cortex of five days old rat (A, B & C are controlled) while (D, E & F are treated) demonstrating:

(A): The cerebellum is more developed with normal appearance of depth of the fissures and lobes (arrows).

(B & C): Notably an increase in the thickness of both external granular layer (E) and molecular layer (M). Differentiated Purkinje cells are arranged in more than one row (P). The dendritic arbor is in a regular pattern (arrowheads) in B with well-developed of the internal granule (I) cells.

(D): Slightly distorted cytoarchitecture of the cerebellum (arrows) with the presence of extensive hemorrhage surround the cerebellum (arrowheads).

(E and F): Note the dispersed inner zone of the external granular layer (E) and decrease in the thickness of the molecular layer (M). Less differentiated Purkinje cells which have poor immature arbors with the irregular arrangement (arrowheads) in E. Multiple cavities in E are clearly noticed (arrow). Focal loss of cells in the molecular, Purkinje and internal granule cells are clearly observed in F (arrow).

(A&D: Hx. &E. x10 240) and (B, C, E&F: Hx. &E.x400).

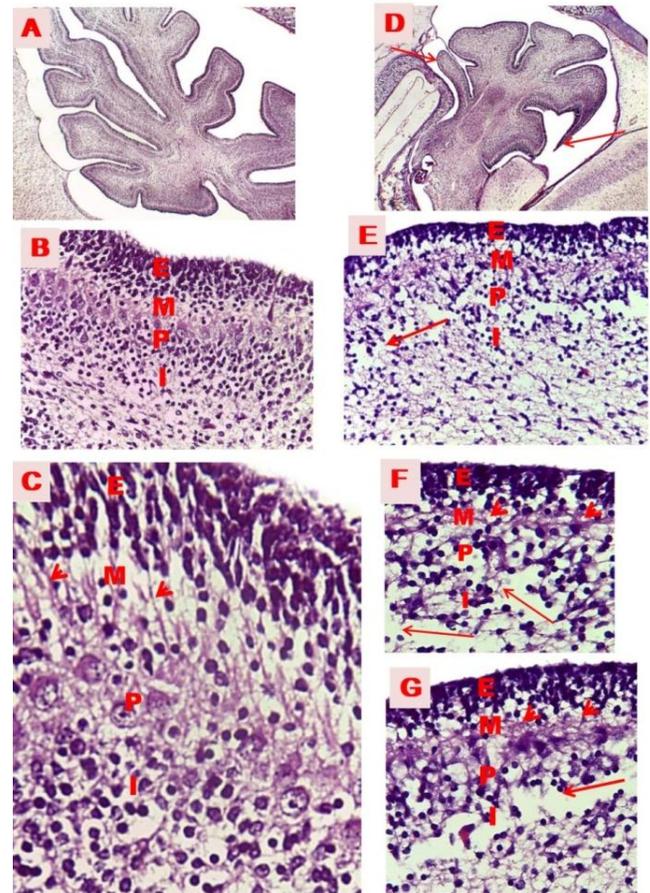


Fig. (4): Photomicrographs of median sagittal sections of the cerebellar cortex of seven days old rat (A, B & C are controlled) while (D, E, F & G are treated) demonstrating:

(A): An increase in the size of the cerebellum. The development and the differentiation of the new fissures are detected.

(B & C): Note the well-developed thick external granular layer (E) and a wide molecular layer (M). Purkinje cells (P) are arranged in a single row parallel to the surface, most of them are aligned in a regular pattern. The parallel dendritic arbors are also clearly seen in C (arrowheads). The well-defined internal granular layer (I) with small rounded cell nuclei can be observed.

(D): Less developed and slightly distorted cerebellum (arrows).

(E & F & G): Note the thin external granular layer (E), moderate focal loss of Purkinje cells (P). Notably, some dendritic arbor degenerate or fuse with adjacent ones in F&G (arrowheads). Notice, persistent distended and fragmented internal granular layer (I) with the presence of vacuolations and cavitation in E, F&G (arrows).

(A & D: Hx&E.x40); (B & E: Hx&E x400) and (C, F&G Hx&E x1000)

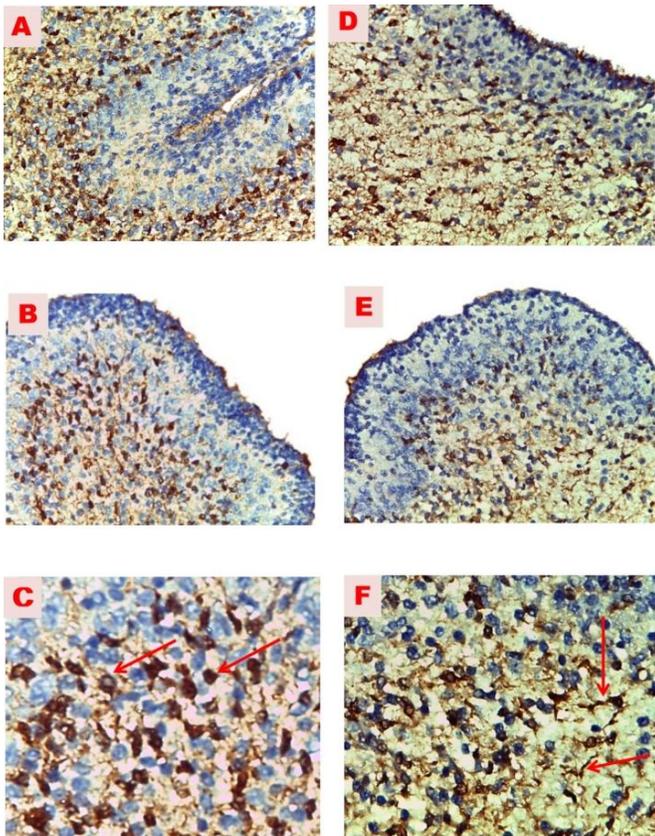


Fig. (5): Photomicrographs of median sagittal sections of the cerebellar cortex of one day old rats (A, B & C are controlled) while (D, E & F are treated) showing:

(A, B, C): Moderate expression of S100 positive immunostaining in the cytoplasm of astrocytes. The normal shape of cells can be clearly seen in C (arrows).

(D, E, and F): Marked expression of S100 immunoreactive astrocytes. Note, the expression is confined to the cytoplasm of astrocytes with enriched few thin irregular processes can be obviously noticed in F (arrows).

C is a higher magnification of B while F is a higher magnification of E.

(A, B, D & E: S100 immunostaining x 400; (C& F: S100 immunostaining x1000).

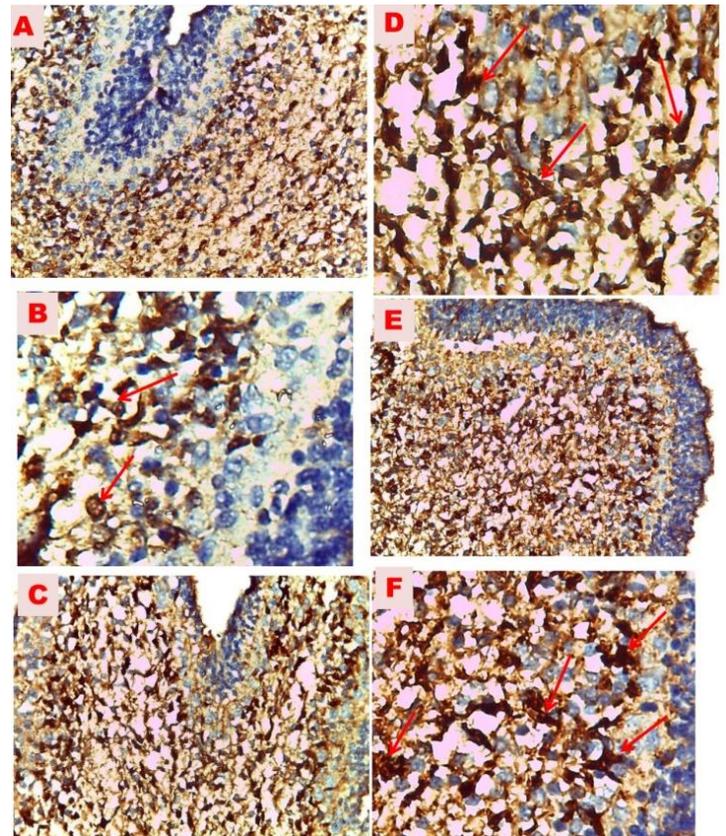


Fig. (6): Photomicrographs of the median sagittal sections of the cerebellar cortex of three days old rats (A& B are controlled) while (C, D, E& F are treated) illustrating:

(A, B): Moderate expression of S100 immunoreactivity in the cytoplasm and their processes. Most of the Astrocytes appear flat rounded with few thin processes in B (arrows).

(C, D, E & F): Marked expression of S100 in astrocytes. Obviously, Astrocytes appear large and irregular with multiple interrupted thick processes and abundant distribution can be clearly detected in D&F (arrows).

D is a higher magnification of C and F is a higher magnification of E.

(A, C and E: S100 immunostaining x 400); (B, D&F: S100 immunostaining x1000).

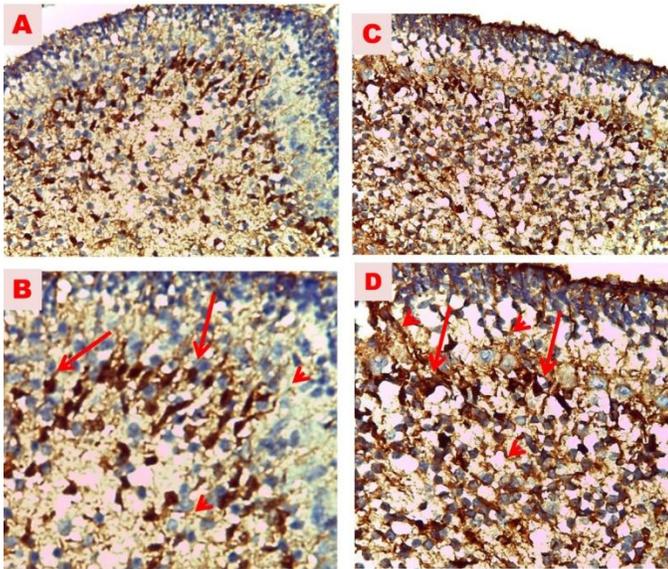


Fig. (7): Photomicrographs of the median sagittal sections of the cerebellar cortex of the five days old rats (A& B are controlled) while (C& D are treated) demonstrating:

(A& B): Moderate positive immunoreaction S100-protein in the cytoplasm of astrocytes. In B the astrocytes appear large (arrows) with thin processes (arrowheads).

(C& D): Marked expression of S100 immunostaining in the cytoplasm of astrocytes and their thick processes. An increase in the size of astrocytes can be clearly seen in D (arrows). The expressions appear abundant in astrocytes and their multiple thick processes in D (arrowheads).

B is a higher magnification of A and D is a higher magnification of C. (A& C: S100 immunostaining x 400) ;(B& D: S100 immunostaining x1000).

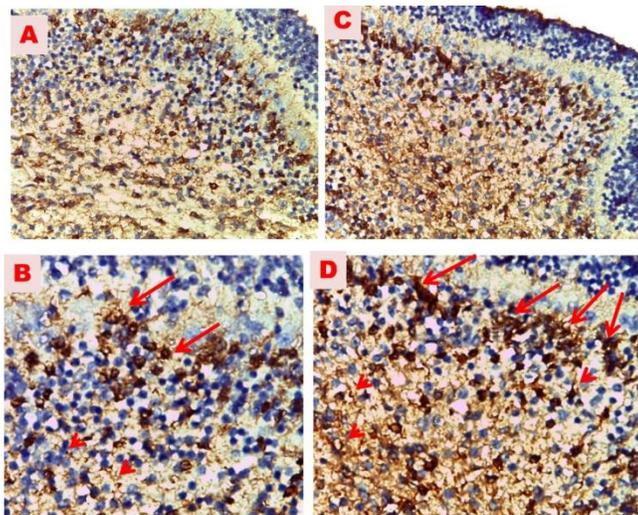


Fig. (8): Photomicrographs of the median sagittal sections of the cerebellar cortex of the seven days old rats (A& B are controlled) while (C& D are treated) demonstrating:

(A& B): Moderate positive cytoplasmic S100 immunoreactivity expression in astrocytes and their few thin processes. In B, Most of the cells appear more or less rounded shape (arrows) with few thin processes (arrowheads).

(C& D): A strong expression of S100 immunostaining in the astrocytes. In D, an increase in their size with disturbed morphology (arrows) and the appearance of thick interrupted processes (arrow heads).

B is a higher magnification of A and D is a higher magnification of C.

(A& C: S100 immunostaining x 400); (B and D: S100 immunostaining x1000).

DISCUSSION

The biochemical results of the current study demonstrated that the pregnant rats treated with Eltroxin and their offspring showed a significant increase in the levels of T3 and T4 and a significant decrease in the level of TSH when compared with their corresponding levels in the pregnant control and their offspring. It is proved that the administration of T4 to adult female rats during pregnancy and lactation periods induced a marked hyperthyroidism in mothers and their offspring. Several studies have shown that maternal hyperthyroidism during pregnancy and lactation leads to a hyperthyroid state in fetus and neonates (Ahmed et al., 2008, 2010; and 2012). The state of hyperthyroidism in fetuses or early neonates is attributed to the passive transfer of maternal T4 from a mother with hyperthyroidism or thyrotoxicosis through the placenta and in mother's milk. Crucially, the decrease in serum TSH level in our results strongly advocated negative feedback effect of the excess circulating THs levels on the anterior lobe of the pituitary gland (Higuch et al., 2005).

In addition, the current work revealed an increase in the oxidative stress activity measured by Malondialdehyde (MDA) levels in the homogenizing fluid of cerebellar tissues from all offspring of treated rats and this also accompanied by a decrease in the antioxidant Glutathione Peroxidase (GPx). In agreement, Messarah et al. (2010) reported that the liver MDA contents rats with experimental hyperthyroidism significantly increased compared with those in the controls. GPx activity was decreased in the muscle, heart, liver and some lymphoid organs in the hyperthyroid rats (Asayama et al., 1987). Moreover, hyperthyroidism increases the metabolic rate that followed by increased oxygen utilization and causes an increase in the production of reactive oxygen species and certainly measurable changes in antioxidative factors (Mayer et al., 2004).

Verity (1994) proposed that reactive oxygen species (R.O.S) represented an important role in the regulation of cell proliferation within the central and peripheral nervous system because reactive oxygen species initiated and stimulated the establishment of neuronal patterns and consequent neurogenesis. Crucially, it was also described that reactive oxygen species played a vital role in physiological processes, but when being in excess reactive oxygen species might cause oxidative damage to molecules (Karbownik and Lewinski, 2003).

The oxidative stress performs a notable role in the pathogenesis of diseases associated with neurodegeneration and accelerated cell death either by apoptosis or necrosis (Bednarek et al., 2004a; Fernández et al., 2006 and Ahmed et al., 2006 and 2012). It may also connect the hyperthyroidism to its sign and symptoms (Guerra et al., 2005). Previous studies had indicated an imbalance between oxidant-antioxidant status and enhanced oxidative stress in hyperthyroidism (Sundaram et al., 1997; Abalovich et al., 2003; Bednarek et al., 2004a, b). Furthermore, in hyperthyroid patients with Graves' disease, an increase in oxidative stress

markers and a decrease in markers of the antioxidant system were detected (Abalovich et al., 2003).

Our results reported alteration, distortion and developmental retardation in the cerebella of the hyperthyroid rat offspring in all examined ages. In addition to the alternation of their oxidative /antioxidative system which proved that the brain exposed to oxidative stress. The results of the present study go hand in hand with Ahmed et al. (2010) who observed an irreversible damage with morphological and cytoarchitecture abnormalities, disorganization, maldevelopment and physical retardation for the central nervous system due to hyperthyroidism during the development. Wong and Leung, (2001) reported that hyperthyroidism in rodents and human disturbed the maturation of the central nervous system and it caused an irreversible dysfunction of the brain if not corrected shortly after birth.

In the current study, the cerebellar cortex consisted of four layers in both the normal and the treated rats offspring from the pial surface inwards, the external granular, the molecular layer, Purkinje layer and the internal granular layer. The current results were in accordance with our previous report by Youssef et al. (2011) in both the normal and treated white rats offspring. As a result of the present study, the cerebellar fissures were shallow and poorly developed in the treated group at 1st postnatal day; furthermore, it appeared more retarded in the treated group at 3rd, 5th and 7th postnatal days. These results were in agreement with Lauder et al. (1974) who reported that hyperthyroidism induced a decrease in the cerebellar foliation in the rats or early maximization of the cortical/subcortical area ratio, which leads to early termination of the foliation procedure.

Furthermore, hyperthyroidism reduced the number of fissures as recorded by Lauder (1978). Mares et al. (1970) and Mares and Lodin (1970) observed that cerebellar foliation is produced from the differential proliferation of the external granular layer. Studies in both rat and mouse cerebella had established that the granule cell precursor proliferation is a necessity for foliation to develop. The experimental reduction of the postnatal rat cerebella by using irradiation or a genetically engineered reduction in granular cell precursor proliferation by mutations in components of the Sonic hedgehog signaling pathway leads to premature diminution of the external granular layer and undeveloped (less complex) foliation form (Doughty et al., 1998 and Corrales et al., 2006).

The present study revealed rapid development in the control offspring during the early postnatal period. These events continued until the end of the first postnatal week. The external granular layer of the control offspring was increased in thickness from the 1st, 3rd, 5th postnatal day and reached the maximum thickness on the 7th postnatal day. These findings received an explicit support from the earlier publication obtained from Noor-El-Din et al. (1977) who stated that the thickness of the external granular layer was increased gradually to reach its maximum at the age of 7 days. In contrast, the current results revealed that the external granular layer in the hyperthyroid rat offspring decreased in thickness in addition to aggregation, clumping and degeneration of some cells throughout the experimental period of offspring treated rats. This finding explains the previously mentioned reduced foliation.

These results were in agreement with Nicholson and Altman (1972a) who suggested that hyperthyroidism caused early termination of cell proliferation in the external granular layer accompanied by early disappearance of this layer. Also,

The existing results were in agreement with the previous publication by Lauder et al. (1974) who reported that hyperthyroidism caused the premature decline and disappearance of the external granular layer. Definitely, hyperthyroidism altered thyroid states and reduced the rate of cell acquisition in the external granular layer (Lauder, 1977a, b).

The molecular layer of the control rat offspring of the present study is grew in breadth from the 1st postnatal day until the age of 7th postnatal day. Similar observation was achieved by (Noor- El-Din et al., 1985). However, the molecular layer of the treated rat offspring was apparently reduced in thickness. Moreover, some of these cells are degenerated and distorted during all the examined periods. Nicholson and Altman (1972a) stated that hyperthyroidism result eventually in a marked deficit in the total number of synapses due to the reduced area of the molecular layer, even though synaptic density was normal. Also hyperthyroidism caused some malformations as terminal decrease in basket cells in rat (Nicholson & Altman, 1972, b, c). Correspondingly, in chick cerebellum, the anticonvulsant drug, phenytoin reduced the thickness of the molecular layer (Allam et al., 1987).

In the current study, Purkinje cells in the control of the 1st postnatal day could be hardly seen they arranged in more than one row intermingled with the cells of the superficial zone of the internal granular layer. They appeared to be larger cells than those of the internal granular layer. The thickness of Purkinje cells increased in the control group of 3rd and 5th postnatal to reach the maximum thickness at the 7th postnatal days, the current results are consistent with Altman and Bayer (1985). In contrast, Purkinje cells of the treated rats were diminished in number with short and thin dendrites in all examined ages. Moreover, some degenerated dendrites and focal loss were also detected. These results were consistent with the Ahmed et al. (2012) who observed that Purkinje cells of offspring of hyperthyroid mothers decreased in number and in their dendrites length and density, and some degeneration was also noticed.

A prominent finding in this study is a reduction in the thickness of the internal granular layer in all ages of the treated animals with deterioration and alteration in its cells. These findings were confirmed by Altman and Winfree (1977). Also, Ahmed et al. (2010) suggested that hyperthyroidism may affect the growth and maturation of neurons through its effect on the vital processes of these neurons. The delayed growth and degeneration of cerebellar neurons and their fibers were associated with an increase in Monoamines levels and ATPase and Ch E activity.

In the present study, the immunohistochemical results might offer a valuable suggestion that astrocytes in the treated rats were moderately altered morphologically. The astrocytes showed an increase in size and number of thick processes. Furthermore, one of the most striking findings was the expression pattern of S100 which was abundant in the astrocytes. Central nervous system trauma or disease are populated by reactive astrocytes with three general properties; the expression of GFAP (astrocyte marker), the cellular hypertrophy, and the cellular proliferation.

Importantly, injury-induced proliferation, hypertrophy, and tiling disorganization are likely to disturb astrocytes functions, and the introduction of new signaling molecules in an injury location raises the question of whether reactive astrocytes after an injury are indicating a loss of normal functions or the gaining of new ones (Burda and Sofroniew, 2014). Thyroid hormone affects the differentiation and maturation of different glial

subtypes including astrocytes, oligodendrocytes, and microglia. Absolutely, astrocytes were the most common cell type in the mammalian brain. The astrocytes play very important functions in the central nervous system (Fields and Stevens-Graham, 2002). Some of that effect of thyroid hormone on neuronal proliferation and differentiation could be mediated by a primary action on astrocytes. In cultured astrocytes, thyroid hormone influenced actin polymerization and integrin–lamina interactions (Siegrist- Kaiser et al., 1990; Farwell et al., 1990, 1995).

The immunohistochemical results stated in the current study might offer a significant suggestion that S100 was highly expressed in the astrocytes of the cerebellum in all developmental ages of 1st, 3rd, 5th and 7th postnatal days of the treated rats offspring. The present results were in accordance with the previous results obtained from Ghandour et al. (1981a, b) and Legrand et al. (1981) they confirmed that in the adults and developing cerebellum S100 protein was detectable exclusively in astrocytes by immunocytochemistry with the light and electron microscopy. Furthermore, these results correspond with those of Farwell and Dubord-Tomasetti (1999) and Alvarez-Dolado et al. (2000) who suggested that thyroid hormones encouraged the expression of astroglial genes. Earlier studies suggested that the developmental pattern of S100 protein, a specific marker of astroglia, was investigated by radioimmunoassay in the cerebellum. Significantly, in the thyroxine treated rats, the total amount and the concentration of S100 protein were higher than in controls during the initial 3 weeks of postnatal life and returned to normal values thereafter (Clos et al., 1982). In contrast, in the rats given an excess of thyroxine from birth, the number of Bergmann astrocytes was not affected at 5 and 35 days whereas that of the internal astrocytes is reduced (Legrand, 1984).

The present study is in parallel with the early in vitro investigation which demonstrated that T3 treatment induced cortical astrocytes presented a flat morphology to become process-bearing cells. T3-treated astrocytes presented a stellate morphology with glial fibrillary acidic protein, GFAP (astrocyte marker) enriched processes (Lima et al., 1997, 1998 and Trentin et al., 1998). Indeed, within a few hours of almost any type of brain injury, surviving astrocytes in the affected region initiated to display hypertrophy and proliferation, termed reactive astrogliosis (Ridet et al., 1997). This response was stimulated by migration of microglia and macrophages to the damaged area. Crucially, reactive astrocytes increased the expression of their structural proteins, GFAP, and vimentin (Eng et al., 2000).

Reactive astrocytes promptly gathered in the damaged area and change their morphology, characteristically inducing swelling. This is associated with osmolarity alterations as a consequence from edema or ischemia following traumatic brain injury (Sofroniew and Vinters, 2010). Astrocytes, like other cells, were susceptible to the reactive oxygen species produced by ischemia–reperfusion (Hollensworth et al., 2000 and Ying et al., 2000).

CONCLUSION

Hyperthyroidism in rats during gestation and lactation periods badly affected the development of cerebellum of the offspring at all examined period, 1st, 3rd, 5th and 7th postnatal days. Hyperthyroidism during pregnancy and lactation periods might cause a number of harmful abnormalities in the development of the cerebellum including disintegration, impairment, and

distortion of neurons and dendrites. Significantly, astrocytes in the offspring of treated rats were reasonably altered morphologically whereas the astrocytes showed an increased number of processes. Thus, further studies need to be done to emphasize this theory. The disturbed cerebellar cytoarchitecture might be due to increase oxidative stress and impaired antioxidant defense system.

FUTURE DIRECTIONS

In view of the initial roles of astrocytes, such as their stem cell properties and contribution in synapse transmission, we might, therefore expect that unique and crucial functions of this cell type regarding thyroid hormone actions on brain development will be discovered in the near future. In addition, because of the various nature and complex biology of astrocytes, and the inadequate number of studies, their role in hyperthyroidism, deserves further study.

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