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LACTATING PTU EXPOSURE: II- ALTERS THYROID-AXIS AND PROOXIDANT-ANTIOXIDANT BALANCE IN NEONATAL CEREBELLUM

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ABSTRACT: *The aim of the present study was to evaluate the effect of lactating 6-propyl-2-thiouracil (PTU) on the interactions between the thyroid-axis and prooxidant/antioxidant markers in neonates. PTU was administered to female rats in drinking water (0.1% w/v) from birth to lactation day (LD) 30. The administration gave rise to a highly significant decrease in serum thyroxine (T4) and triiodothyronine (T3) levels and increase in serum thyrotropin (TSH) level in both dams and their offspring at LDs 20 and 30 relative to control group. Also, in PTU group, a marked depression was observed in serum of neonatal growth hormone (GH). In this hypothyroid state, obvious elevation of oxidative markers (protein carbonyls, lipid peroxidation, NO and H_2O_2) was observed at postnatal days (PNDs) 20 and 30, along with decreased activities of antioxidants markers (total thiol, glutathione, glutathione peroxidase, superoxide dismutase and catalase) in neonatal cerebellum with respect to control group. Also, the administration caused some histopathological changes in neonatal cerebellar cortex, such as oedema, vacuoles, reduction of the Purkinje cells, and cellular fragmentations at PND 30. Thus, hypothyroidism in lactating PTU impairs the neonatal neuroendocrine system via production of free radicals, and this, may cause damage of proteins and lipids at the plasma membrane and cellular components delaying cerebellum development.*

KEYWORDS: 6-propyl-2-thiouracil, Thyroid, Cerebellum, Antioxidants, Prooxidant, Rat newborns

INTRODUCTION

In the last fifteen years, an increasing number of studies have indicated that THs have important physiological functions, not only during brain maturation (Ahmed and Incerpi, 2013) but also in the adult vertebrate brain (Broedel et al., 2003; Horn and Heuer, 2010). THs are known to set the cellular basal metabolic rate and are considered as major regulators of energy metabolism; mitochondrial activity and biogenesis; oxygen consumption and active oxygen metabolism (Martinez et al., 2001; Bhanja and Chainy, 2010). Thus, one of the most important functions performed by THs is the tight regulation of cellular oxygen consumption and consequent generation of reactive oxygen species (ROS) in several tissues (Mircescu, 2008; Petrulea et al.,

2009) including brain (Rahaman et al., 2001) that can be attacked by the ROS and start lipid peroxidation (LPO) (Garcia et al., 2005; Valko et al., 2007). In addition, the brain processes a great amount of oxygen per unit tissue mass as it consumes 20% of the entire oxygen consumed by the body though its weight is only 2% of the total body weight (Halliwell, 2006); more so in the postnatal period due to higher brain to body ratio. Generally, ROS generation is controlled by enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and non-enzymatic antioxidants such as total thiol (t-SH) and glutathione (GSH) (Ahmed et al., 2013; Al-azzawie et al., 2013; Imosemi, 2013). These mechanisms involve preventive mechanisms, repair mechanisms, physical and antioxidant defences. Interestingly, ROS are known to play important roles in regulating proliferation and differentiation (Hancock et al., 2001; Valko et al., 2007).

6-propyl-2-thiouracil (PTU) is an anti-thyroid drug which inhibits both the synthesis of THs in the thyroid gland, and the conversion of T4 to its active form, T3, in peripheral tissues (Gilbert, 2011; Santos-Ahmed et al., 2011; Chang et al., 2012; Fujimoto et al., 2012; Bhanja and Jena, 2013; Hassan et al., 2013). The deficiency in THs during the developmental period may result in an irreversible impairment, morphological and cytoarchitecture abnormalities, disorganization, maldevelopment and physical retardation that are permanent (Zoeller and Rovet, 2004; Zoeller and Crofton, 2005; Argumedo et al., 2012; Hassan et al., 2013). More so, there are disturbances in the balance between the ROS generation and antioxidant defence system in most developing brain regions due to the hypothyroidism by anti-thyroid drugs (Ahmed et al., 2012; Bhanja and Jena, 2013). These effects can produce an increase of oxidant species that causes lipid peroxidation (LPO), nitration, carbonylation, or glutathionylation of proteins, and fragmentation of DNA (Halliwell and Gutteridge, 2007; Valko et al., 2007).

Since PTU severely affect the growth of the young offspring (Argumedo et al., 2012), the aim of this study was to examine the effects of lactating PTU on the developmental aspects of the thyroid markers in rat dams and their newborns. Additionally, the current study extends not only to view the changes in the histogenesis of the cerebellar cortex, but also to follow the changes in the activities of prooxidant/antioxidant markers at PNDs 20 and 30. In this regard, cerebellum was used as a model system, because this region is highly sensitive to any stress (TH disturbance) (Koibuchi et al., 2001; Koibuchi, 2009; Ahmed, 2011) where its development occurs postnatally in the rat (Altman and Bayer, 1997). Also, this developmental stage in the rat reflects the time period between the third trimester of gestation to the second postnatal year in the human (Ahmed et al., 2008).

MATERIALS AND METHODS

I- Animals and treatments:

Pregnant white albino rats (*Rattus norvegicus*) at gestation day 15 were purchased from the National Institute of Ophthalmology, Giza, Egypt. They were housed singly in cages until giving birth, after which litters were culled to 8 pups per cage so that mothers could provide sufficient milk for the pups. The male litters only were randomly divided into control and PTU groups. The animals were fed on standard rodent pellet diet manufactured by the Egyptian Company for oil

and soap as well as some vegetables as a source of vitamins (Ahmed et al., 2010; El-bakry et al., 2010). Tap water was used for drinking *ad libitum* and these animals were exposed to constant daily light/dark periods of 12 h each (lights on at 06:00 h) and 50 ± 5% relative humidity (Ahmed and Incerpi, 2013). Generally, all the animal procedures were in accordance with the general guidelines of animal care and the recommendations of the Canadian Council on Animal Care (CCAC; Olfert et al. 1993). All efforts were made to minimize the number of animals used and their suffering.

0.1% w/v PTU (Koohestani et al., 2012) (Sigma- Aldrich, St. Louis, MO, USA) was administered to female rats in drinking water from birth to lactation day (LD) 30, which was replaced daily with fresh PTU solution. PTU enters the bloodstream, passing through the mother's breast milk, to the rat pups. For each time point, age-matched control rats received normal drinking water. At LDs 20 and 30, dams and their newborns were subsequently sacrificed under mild diethyl ether anaesthesia, and the blood samples from dams and their pups were taken and centrifuged at 1006.2 xg for 30 min. The neonatal cerebellums were placed immediately in ice-cold normal saline. The clear, non-hemolysed supernatant sera were quickly removed, divided into three portions for each individual animal, and kept at -30 °C until use for hormonal examinations. The cerebellums were homogenized by using a Teflon homogenizer (Glas-Col, Terre Haute, USA) in ice-cold PBS (0.15M, pH 7.4) containing 0.25 M sucrose and 2mM EDTA for antioxidants and oxidataive markers determinations. The supernatants were kept in a deep freezer at -30 °C until use.

The cerebellums of the offspring were removed immediately after a rapid anaesthesia, dropped into the 10% neutral buffer formalin as a fixative for general histological structure (haematoxylin and eosin stain; Bancroft and Stevens, 1982). All sections were evaluated for the degree of any injury.

II- The radioimmunoassay (RIA) examination:

T4, T3 and TSH in serum of mothers and their offspring, as well as GH in serum of the newborns were estimated quantitatively in the Diabetic Endocrine Metabolic Pediatric Unit, Center for Social and Preventive Medicine, New Children Hospital, Faculty of Medicine, Cairo University, Egypt according to the method of Thakur et al. (1997), Maes et al. (1997), Mandel et al. (1993) and Reutens (1995), respectively. The kits were obtained from Calbiotech INC (CBI), USA.

III- Histological examination in neonatal cerebellum:

Some cerebellum samples, intended for histological examination by light microscopy, were immediately fixed in 10% of neutral buffer formalin and processed in a series of graded ethanol solutions. They were then embedded in paraffin, serially sectioned at 6 μm and stained with hematoxylin-eosin in Histology and Histopathology Unit, Faculty of Veterinary Medicine, Beni Suef University, Egypt.

IV- The developmental and biochemical examinations in neonatal cerebellum:

A- Determination of antioxidant markers:

1- Total thiol (t-SH) concentration:

t-SH was determined according to the method of Koster et al. (1986). The reagents were prepared in the laboratory. The method was based upon the reduction of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid; DTNB) by thiols containing compounds to form 5-thio-2-nitrobenzoic acid, which can be measured colorimetrically at 412 nm. The aliquot of the supernatant (0.25 mL) was mixed with 0.75 mL phosphate buffer and 0.2 mL Ellman's reagent. Mixing was followed by incubation for 5 min at 37 °C. Distilled water instead of the sample was used for blank preparation. Absorbance was measured at 412 nm against blank. The following equation was used for calculation of total thiols concentration in nmol/100mg tissue: Thiols concentration= (Absorbance×20×10^6×5×1000) / (0.5×13600×10); where 13600 was the molar extinction coefficient standardized and calculated by Beutler et al. (1963).

2- Glutathione (GSH) concentration:

The method is based on the development of a yellow color when DTNB is added to compounds containing sulfhydryl groups (Jollow et al., 1974). Supernatants in phosphate buffer (500 µL) were added to 3 mL of 4% sulfosalicylic acid. The mixture were centrifuged at 1600 ×g for 15 min. Supernatants (500 µL) were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. The GSH concentration was expressed as nmol/100mg tissue.

3- Glutathione peroxidase (GPx) activity:

GPx was determined according to the method of Pinto and Bartley (1989). The reagents prepared in the laboratory. The GPx activity was estimated by the so-called "chemical" method in the presence of hydroperoxide (R–O–OH) and GSH. In this case, we estimated the residual GSH remaining after the action of enzyme in the presence of DTNB or Ellman's at 412 nm (Sedlak and Lindsay, 1968). One unit of GPx converts 1 µmole of GSH into GSSG/min at 30 °C at pH 7.4.

4- Superoxide dismutase (SOD) activity:

SOD was measured using pyrogallol as substrate (Shukla et al., 1987). This method follows the superoxide-driven auto-oxidation of pyrogallol at pH 8.2 in the presence of EDTA. The assay mixture contained 1 mM EDTA in 50 mM Tris-HCl buffer (pH 8.2) with or without the sample. The reaction was started by the addition of pyrogallol (final concentration 0.124 mM), and the oxidation of pyrogallol was followed for 1 min at 420 nm. The percent inhibition of the auto-oxidation of pyrogallol by SOD present in the tissue sample was determined. One unit (U) of SOD activity was defined as the amount that reduced the absorbance change by 50% (U/mg protein). Copper/zinc SOD (SOD1) was differentiated from manganese SOD (SOD2) by addition of 2 mM sodium cyanide to inhibit the activity of SOD1 from total SOD activity. SOD1 activity was calculated as the difference between total SOD and SOD2 activity as in a previous report (McIntosh et al., 1998).

5- Catalase (CAT) activity:

CAT was determined using reagents prepared in the laboratory (Cohen et al., 1970). The CAT was determined by using a relative low hydrogen peroxide concentration to avoid inactivation of the enzyme during assay (usually 30 second) or formation of bubbles in the cuvette due to liberation of O_2. The H_2O_2 concentration is critical in as much as there is direct proportionally between the substrate concentration and the rate of decomposition. The remained H_2O_2 reduced the titrated $KMnO_4$ color which was measured at 480 nm. The activity of enzyme was expressed in terms of the 1^{st} order rate constant (k) which was calculated as follows: $k = \text{Log}(S_0/S_3) \times 2.3/t$, where, K is the 1^{st} order reaction rate constant, t is the time interval over which the reaction

was measured (viz., 3), S_0 is the subtract of the absorbance of the reaction system blanks (B) from the spectrophotometric standard and S_3 is the subtract of the absorbance of the reaction samples (A) from the spectrophotometric standard.

B- Determination of prooxidant markers:

1- Protein carbonyl (PCa) content:

PCa content was measured with the method of Reznick and Packer (1994). Briefly, 100 µL of the supernatant was placed in glass tubes. Then 500 µL of 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH) in 2 mol/L HCl was added. Tubes were incubated for 1 h at room temperature. Samples were vortexed every 15 min. Then 500 µL of trichloroacetic acid (TCA) (20%) were added and the tubes were left on ice for 5 min followed by centrifugation for 10 min. The pellet was then washed twice with ethanol-ethyl acetate (v/v). The final precipitate was dissolved in 600 µL of 6 mol/L guanidine hydrochloride solution and incubated for 15 min at 37 °C. The absorbance of the sample was measured at 370 nm. The PCa content was expressed as µmoles/mg protein.

2- Malondialdehyde (MDA) level:

The MDA concentrations, index of LPO, were determined spectrophotometrically according to Draper and Hadley (1990). Briefly, supernatant was mixed with 1 mL of 5% TCA and centrifuged at 2500 ×g for 10 min. An amount of 1 mL of thiobarbituric acid (TBA) reagent (0.67%) was added to 500 µL of the supernatant and heated at 90 °C for 15 min. The mixture was then cooled and measured for absorbance at 532 nm. The MDA values were calculated by using 1,1',3,3'-tetraethoxypropane as standard and expressed as nmoles MDA/100mg/hr.

3- Nitric oxide (NO) level:

The amount of NO generated was measured using Griess reaction (Dutta et al. 2008). Briefly, equal volumes of supernatant, sulfanilamide and N-1-napthylethylenediamine dihydrochloride were incubated at 25 °C for 5 min, and the formation of an azocompound was measured colorimetrically at 550 nm. The concentration of NO was extrapolated from a standard curve of sodium nitrite.

4- Hydrogen peroxide (H_2O_2) content:

The H_2O_2 content was determined according to Sergiev et al. (1997). 0.5 mL of the supernatant was added with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide, and the absorbance was measured at 390 nm. H_2O_2 was used as a standard and expressed as nmol H_2O_2/g tissue.

V- Statistical analysis:

The concentration of the parameters studied in the blood serum and in the neonatal cerebellum was expressed as mean ± S.E. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by the LSD test to discern the main effects and compare various groups with each other. All calculations were performed using PC-STAT statistical software (University of Georgia, 1985). F-probability for each variable expresses the general effect between groups. Values of $P<0.01$ and $P<0.001$ are considered statistically highly significant and very highly significant, respectively.

RESULTS

I- Maternal-neonatal thyroid markers:

In control rats, the levels of T3, T4 and TSH in both dams and their newborns, and neonatal GH showed an age related increase from LD 20 to LD 30 (Figures 1 & 2). T3 and T4 levels showed a

highly significant (P<0.01) decrease in PTU-treated dams at LDs 20 and 30 with respect to control group (Figure 1). In particular the level of T3 at LD 30 was lower than LD 20. A highly significant increase in serum TSH level was seen in PTU-treated dams at both LDs as compared to control group (Figure 1). On the other hand, the maternal administration caused a highly significant (P<0.01) decrease in the levels of serum T4, T3 and GH of neonatal rats at PNDs 20 and 30 if compared to their control group (Figure 2). However, the level of serum TSH of neonatal rats showed a highly significant age related increase in PTU-treated dams (P<0.01). Concerning one way ANOVA for all tested hormones, it was revealed that the general effect between both groups was very highly significant (P<0.001) in both dams and their pups throughout the experimental period.

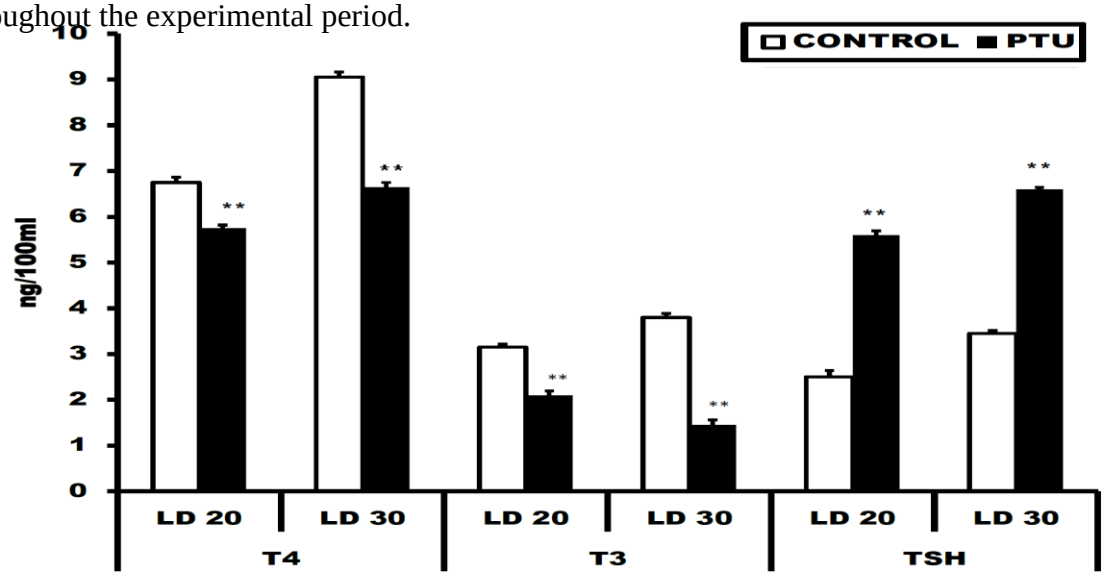

Figure 1. Levels of T4, T3 and TSH (expressed in ng/100ml) in the serum of the control and PTU-treated dams during the lactation period. Bars represent mean ± SE of six animals/group, where the change between both groups/PND is highly significant (P < 0.01) as determined by LSD test. ANOVA (F-probability) expresses the effect between groups and tested PNDs, where P < 0.001 is very highly significant.**

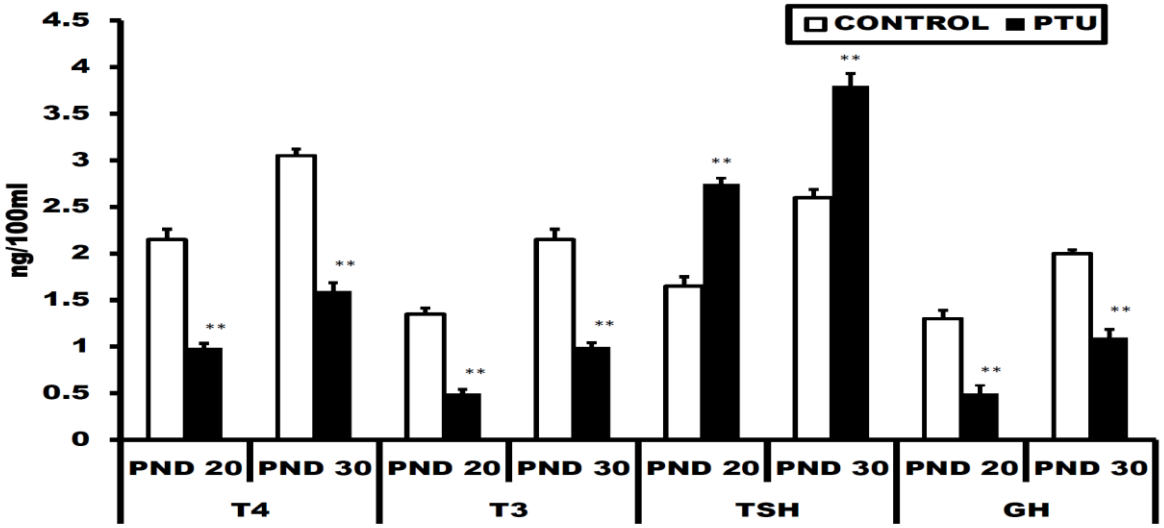

Figure 2. Effect of maternal PTU in serum thyroid markers (T4, T3, TSH and GH; expressed in ng/100ml) of their newborns during the lactation period. Bars represent mean ± SE of six animals/group, where the change between both groups/PND is highly significant (P < 0.01) as determined by LSD test. ANOVA (F-probability) expresses the effect between groups and tested PNDs, where P < 0.001 is very highly significant.**

II- Histoarchitecture and histopathological changes in the cerebellar cortex:

In control group, the molecular layer (ML) was shown located internally before the Purkinje cell layer (Figure 3A). The Purkinje layer (PL) comprised pear-shaped neurons and was arranged in a single row at a junction of the ML and internal granular layer (IGL) at PND 30 (Figure 3A). A large, rounded nucleus was evident in each cell body of the Purkinje cells (PCs) with thick remarked cytoplasmic coat at this day. Also, the IGL was shown situated just below the PC layer and it was extremely cellular and formed of closely packed oval or rounded shaped neurons with the age progress. These neurons were infiltrated with intercellular spaces called island or glomeruli. The neurons of this layer had a large, darkly stained nuclei enclosed with a thin peripheral cytoplasmic coat (Figure 3A).

On the other hand, the maternal PTU-induced hypothyroidism, from LD 20 to 30, led to marked histopathological lesions in the cortex of their newborns. These lesions appear in the form of oedematous areas that were observed in the IGL with some cellular fragmentations in the PL and IGL (Figure 3B). Also, vacuolation in ML and reduction in the size and numbers of the PCs were observed at PND 30. In general, neuronal cells, in all cerebellar cortex layers, suffered from severe distortions (degenerative changes), particularly in PL and IGL (Figure 3B).

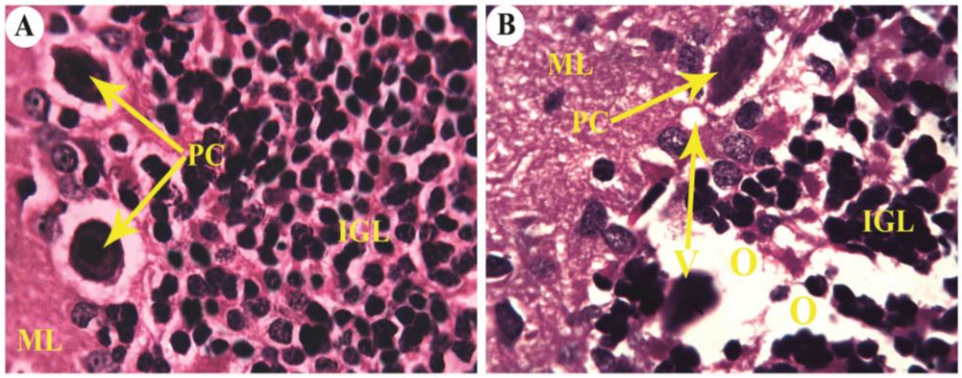

Figure 3. Representative HE-stained photomicrographs (X1000) of neonatal cerebellar cortex at PND 30 (A: Control; B: PTU). Where, IGL is internal granular layer, ML is molecular layer, O is oedema, PC is Purkinje cell and V is vacuoles.

III- Developmental and biochemical markers in neonatal cerebellum:

Tables 1 and 2 show the changes in non-enzymatic antioxidants (t-SH and GSH), enzymatic antioxidants (GPx, SOD1, SOD2 and CAT) and oxidative stress markers (PCa, LPO, NO and H_2O_2) of neonatal cerebellum, whose mothers were hypothyroid by PTU from birth to LD 30. In control newborns, these biochemical variables were gradually increased with ageing from PND 20 to 30, and the administration of maternal PTU disrupted this behavioral pattern. This administration induced a highly significant ($P<0.01$) depletion in the values of the t-SH, GSH, GPx, Mn-SOD, Cu/Zn-SOD and CAT at PND 20 in comparison with the control ones (44.74±0.687, 23.18±0.934, 9.65±0.163, 2.78±0.060, 3.66±0.146 and 9.99±0.122 vs 95.08±0.213, 36.61±0.716, 13.65±0.200, 4.25±0.116, 5.12±0.388 and 13.50±0.174, respectively) (Table 1). Moreover, the most potent decreased effect of PTU on t-SH (-49.98%), GSH (-32.38%), GPx (-74.80%), Mn-SOD (-72.95%), Cu/Zn-SOD (-72.38%) and CAT (-60.06%) was found at PND 30. Conversely, the PTU administration exhibited the reverse pattern of changes; the increase in PCa (+112.42%), MDA (+197.15%), NO (+197.87%) and H_2O_2 (+214.28%) levels was highly significant (LSD; $P<0.01$) at PND 20 with respect to control group. This increase was more significant at PND 30; the percentage change was +148.72%, +298.13%, +258.57% and +618.18% for PCa, MDA, NO and H_2O_2, respectively (Table 2). ANOVA indicated that the general effect between both groups was found to be very highly significant ($P<0.001$) during the experimental period (Tables 1 and 2).

Table 1. Effect of maternal PTU in antioxidant markers [t-SH (nmol/100mg), GSH (nmol/100mg), GPx (mU/100mg), Mn-SOD & Cu/Zn-SOD (U/mg), and CAT ($K.10^{-2}$)] of neonatal cerebellum during the lactation period. Data are expressed as mean ± SE. Number of animals in each group is six. Values which share the same superscript symbols are not significantly different. ANOVA (F-probability) expresses the effect between groups and tested PNDs, where P<0.001 is very highly significant.

Periods	PTU	t-SH	GSH	GPx	Mn-SOD (SOD2)	Cu/Zn-SOD (SOD1)	CAT
PND 20	0	95.08±0.213^{b}	36.61±0.716^{b}	13.65±0.200^{b}	4.25±0.116^{b}	5.12±0.388^{b}	13.50±0.174^{b}
	0.1% w/v	44.74±0.687^{d}	23.18±0.934^{d}	9.65±0.163^{c}	2.78±0.060^{c}	3.66±0.146^{c}	9.99±0.122^{c}
		-52.94%	-36.68%	-29.30%	-34.58%	-28.51%	-26.00%
PND 30	0	102.08±0.140^{a}	42.83±0.999^{a}	18.85±0.161^{a}	7.10±0.096^{a}	9.09±0.119^{a}	15.40±0.144^{a}
	0.1% w/v	51.06±0.359^{c}	28.96±0.460^{c}	4.75±0.290^{d}	1.92±0.070^{d}	2.51±0.183^{d}	6.15±0.119^{d}
		-49.98%	-32.38%	-74.80%	-72.95%	-72.38%	-60.06%
ANOVA				P<0.001			
LSD 5%		3.937	2.436	0.642	0.690	2.410	0.318
LSD 1%		5.369	3.321	0.876	0.940	3.288	0.433

Table 2. Effect of maternal PTU in oxidative stress markers [PCa (µmoles/mg), MDA (nmoles MDA/100mg/hr), NO (µmol/ml) and H_2O_2 (nmol/g)] of neonatal cerebellum during the lactation period. Data are expressed as mean ± SE. Number of animals in each group is six. Values which share the same superscript symbols are not significantly different. ANOVA (F-probability) expresses the effect between groups and tested PNDs, where P<0.001 is very highly significant.

Periods	PTU	PCa	MDA	NO	H_2O_2
PND 20	0	8.45 ± 0.118^{c}	9.50 ± 0.134^{c}	2.35 ± 0.110^{d}	0.70 ± 0.047^{c}
	0.1% w/v	17.95 ± 0.951^{b}	28.23 ± 0.988^{b}	7.00 ± 0.179^{b}	2.20 ± 0.087^{b}
		+112.42%	+197.15%	+197.87%	+214.28%
PND 30	0	10.55 ± 0.151^{c}	10.70 ± 0.137^{c}	3.25 ± 0.074^{c}	0.55 ± 0.221^{c}
	0.1% w/v	26.24 ± 0.899^{a}	42.60 ± 0.996^{a}	12.30 ± 0.491^{a}	3.95 ± 0.157^{a}
		+148.72%	+298.13%	+258.57%	+618.18%
ANOVA		P<0.001			
LSD 5%		3.937	0.260	0.795	0.275
LSD 1%		5.369	0.356	1.085	0.376

DISCUSSION

In view of thyroid function of control dams and their newborns, the current study revealed gradual increases of serum T4, T3 and TSH levels at LDs 20 and 30. Also, neonatal GH was markedly elevated in an age-dependent manner from PND 20 to PND 30. These results agreed with several previous experiments (El-bakry et al., 2010; Ahmed et al., 2010 & 2012; Ahmed, 2011). Also, the level of neonatal GH (Zimmermann, 2011; Ahmed, 2012) was increased steadily during the first 3rd postnatal week (Ahmed et al., 2012).

Our data are in quite good agreement with previously published literature (Bhanja and Chainy, 2010) as far as alterations of THs, TSH and GH and antioxidant defence system (GSH, GPx, SOD1, SOD2 and CAT) are concerned in both hypothyroid mothers and newborns. At variance with their results we found at LD 30 a decrease of T3, instead of an increase, with respect to LD 20, as expected with an age-dependent change. In addition to these data, we also show changes of nitric oxide production and H_2O_2 and more damage due to PTU-treatment, resulting from both protein and lipid damage that may be responsible in the long term range of alterations in cerebellum and CNS development.

The present study revealed that administration of PTU in drinking water (0.1%, w/v) to adult female rats during the lactation period induced hypothyroidism in mothers and their newborns as indicated by a decrease in serum T4 and T3 levels, and an increase in serum TSH level at LDs 20 and 30. The maternal administration decreased the level of neonatal GH at both tested PNDs as compared to control group. Similarly, PTU produced a step-wise decrease in serum total T4, and a step-wise increase in serum TSH (Sharlin et al., 2009). This hypothyroid state is caused by inhibition of thyroperoxidase activity, blocking the organification of iodine and the synthesis of THs (Jena et al., 2012; Tousson et al., 2012; Gilbert and Lasley, 2013; Hassan et al., 2013; Khalawi et al., 2013). The reported lower value of T3 at LD 30, with respect to LD 20 could be due to an impairment of 5'-deiodinase (Manna et al., 2013). Also, this thyroid dysfunction appears to be closely related to ROS formation as reported by Ahmed et al. (2012) and as will be explained below. These potential effects result in impaired growth of the young offspring and imply that maternal PTU may act as a disruptor for the developmental pituitary-thyroid axis (PTA) (Axelstad et al., 2008; Argumedo et al., 2012).

On the other hand, the stepwise progress in the levels of THs during this period may be necessary for normal cerebellum development as observed in this study. Consistent with these data, THs in rat mainly affect cerebellar development during the first 2-3 weeks of postnatal life (Wang et al., 2012; El-Bakry et al., 2010; Ahmed, 2012) and is required for normal maturation of this region (Koibuchi, 2006; Bernal, 2007; Ahmed, 2011; Ahmed and Incerpi, 2013). Also, Koibuchi (2013) emphasized that the genomic and non-genomic actions of the T3 and T4 play a critical role in cerebellar development.

There were deformations and developmental defects in the cerebellar cortex of the present hypothyroid rat newborns with the age progress (PND 30), such as oedematous areas in the IGL, vacuoles in ML, reduction in the size and number of the PCs, and fragmentation of the cellular element of the PL and IGL. Concurrently, PTU-treated rats show growth retardation, a reduction in cerebellar mass, and alterations in cerebellar structure, which are characterized by delayed migration of granular cells, short PC dendritic arborization, and a significant reduction in the number of synaptic connections (Lavado-Autric et al., 2003, Li et al., 2004). Particularly, PTU-induced hypothyroidism has been shown to increase apoptosis in the cerebella on PNDs 7 and 30 (Bhanja and Chainy, 2010). Generally, the developing cerebellum is vulnerable to TH deficiency (Anderson, 2008; Koibuchi, 2009: Ahmed, 2011).

To further establish the effect of maternal PTU on the developing cerebellum, we studied its effect on the prooxidant/antioxidant markers. In the control group, the levels of antioxidant markers (t-SH, GSH, GPx, SOD1, SOD2 and CAT), protein oxidation marker (PCa), LPO marker (MDA), NO and H_2O_2 markers of neonatal cerebellum followed a synchronized course of development from PND 20 to 30. More recently, the enzymatic and non-enzymatic antioxidant defence variables (SOD, CAT, GPx, t-SH and GSH) and LPO in the control rat offspring were substantially and gradually increased from the first to the third week old in most brain regions (Ahmed, 2012; Ahmed et al., 2006 & 2012). As well, the prooxidant/antioxidant balance and detoxification of potentially damaging ROS was crucial for cellular homeostasis (Livingstone,

2001; Ahmed, 2012; Ahmed et al., 2012), and may play an important role in a healthy life for the newborns (Ahmed et al., 2013). Thus, based on this evidence, higher levels of cerebellar enzymatic and non-enzymatic antioxidant markers in the control rats detected at PND 30 may be important in protecting the neonatal brain from H_2O_2 or NO toxicity. This balance or protection may be mediated by the thyroid states of both dams and their newborns. These investigations were in accordance with those of Das and Chainy (2001), Dasgupta et al. (2007) and Ahmed et al. (2008 & 2012). The most surprising result of the present study concerned the modulation between THs and GSH in developing cerebellum of the control group. These investigations are in accordance with those of other observations; TH is important in maintaining the GSH homeostasis in the developing rat brain (Ahmed et al., 2008 & 2010). Also, data recorded herein support a positive association between the production of SOD, CAT and H_2O_2 with the THs and TSH levels in the control group. Under normal conditions, specific membrane-bound nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase (DUOX complex) provides H_2O_2 for THs synthesis (Ahmed, 2012; Petrulea et al., 2012), THs modulate the action of SOD and CAT on the H_2O_2 formation and breakdown (Das and Chainy, 2001), and TSH stimulates the organification of iodine by the increase in the production of H_2O_2 (Petrulea et al., 2012). From this, it can be inferred that the production of these markers is synergistic and closely interrelated with the behavior of the PTA during the postnatal period. Accordingly, hypothalamic-pituitary-thyroid axis (HPTA) regulates metabolism and growth, and brain development (Lema et al., 2009).

The maternal administration of PTU through drinking water resulted in a significant increase in LPO and protein oxidation of neonatal cerebellum as indicated by the significant increase in MDA, PCa, NO and H_2O_2 levels at PNDs 20 and 30, suggesting that PTU activated the formation of free radicals (oxidative stress state) during this period. In addition, the inhibition of enzymatic and non-enzymatic antioxidant defence systems (t-SH, GSH, GPx, SOD1, SOD2 and CAT) was observed at both examined PNDs. These alterations were more pronounced at PND 30. This disturbance may lead, in turn, to the malfunction of the developing cerebellum.

Similarly, a progressive hypothyroidism during the postnatal rat brain development led to a decline in GSH level (Rahaman et al., 2001; Mogulkoc et al., 2005; Ahmed, 2012), t-SH level and GPx activity (Ahmed, 2012) associated with an increase in H_2O_2 (Bhanja and Chainy, 2010), PCa (Rahaman et al., 2001), LPO (Rahaman et al., 2001; Bhanja and Chainy, 2010) and hydroxyl radical ($^{\cdot}OH$) levels (Rahaman et al., 2001; Ahmed et al., 2008). These disorders are associated with augmented oxidative stress (Dasgupta et al., 2005; Ahmed et al., 2012) and are consistent with those reported in the present study. More interestingly, Manna et al. (2013) report that PTU decreases GSH level by inhibiting the activity of 5'-deiodinase to convert T4 to T3. Worth noting in the present study that low neonatal GSH content also can be correlated with low GPx activity and this association may produce increased oxidative stress propensity and depletion of the antioxidant capacities as a result of maternal administration. Oxidative stress as a result of PTU treatment may initiate a cascade of events that result in cellular ionic imbalance, signal transduction, and enzyme activity modifications in mammalian CNS (Janaky et al., 1999). Also, peroxidation of membrane lipids can alter membrane structure, membrane fluidity and cell

compartmentalization, which may contribute to impaired cellular function and necrosis (Reiter et al., 2001).

More recently, Mancini et al. (2013) show low total antioxidant capacity levels in hypothyroid patients. The reduction in SOD1 and SOD2 herein is compatible with the reports of Bhanja and Chainy (2010) and Bhanja and Jena (2013) with different PNDs, where a decreased SOD1 and SOD2 expression in cerebellum was found in neonatal hypothyroid rats at 7, 15 and 30 days. The results of the present study suggest that SOD2 is more sensitive to persistent hypothyroidism than SOD1. It is unknown whether these influences occur proportionally. Kono and Fridovich (1982) demonstrated that a decrease in SOD activity might lead to accumulation of superoxide radicals ($O_2^{\cdot-}$), which in turn inhibit CAT activity. An alternative attribution for the present findings is that the high levels of H_2O_2 are also known to inactivate the CAT (Lardinois et al., 1996) and increase tissue sensitivity to oxidant injury (Ho et al., 2004). Generally, protein oxidation can lead to a loss of critical thiol groups in addition to modifications of amino acids leading to the formation of carbonyl and other oxidized moieties (Kehrer, 1993; Petrulea et al., 2012). This disturbance has deleterious effect on the health of the newborns and adulthood (Ahmed et al., 2013). Results of the current investigation suggest that the compensatory increase in neonatal antioxidant markers may be a trial from the antioxidant defence system to acclimatize the new condition in which excess of oxidative markers are produced due to the administration of lactating PTU. These dysregulations may confirm the presence of free radical damage and oxidative stress in neonatal cerebellum of PTU-hypothyroid group. These findings raise the possibility that the imbalance between prooxidant and antioxidant systems in the PTU-hypothyroid group may induce the dysfunction in neonatal thyroid-brain axis.

It is of relevance to correlate altered levels of ROS in the current hypothyroid state with the histopathological lesions in the neonatal cerebellar cortex and the reverse is true. This supports the hypothesis that decreased TH may be a relevant predictor for long-lasting developmental neurotoxicity. PTU-induced hypothyroidism was reported by Singh et al. (2003) and Bhanja and Chainy (2010) to induce oxidative stress in rat cerebellum that resulted in tissue damage and apoptosis. Also, the LPO is involved in the damaging mechanism of several acute and chronic brain disorders of rats (Garcia et al., 2005; Venditti and DiMeo, 2006; Messarah et al., 2007 & 2010), where its destruction can lead to cell death (López-Torres et al., 2000; Das and Chainy, 2001). As well, the variations in total antioxidant defences in developing rat brain may predispose structures to oxidative stress-related neurodegenerative disorders (Siqueira et al., 2005; Kolosova et al., 2006) and cell death depending on the region of the brain affected and the severity of the insult (Ferriero, 2004).

CONCLUSION

Four conclusions are obvious from these data. (1) The proper level of THs during the normal development is both necessary and stimulatory for the proper postnatal growth and development of the cerebellum. (2) The lactating PTU seems to cause dyshormonogenesis (hypothyroid state) and disrupt the development of TH-GH axis. (3) The hypothyroidism by lactating PTU could delay the maturation of the neonatal neuroendocrine system, and this deficit may be due to the

production of free radicals, and reduction the antioxidant defence system. This, in turn, may alter cellular structures, such as proteins and lipid of the plasma membrane and different cell compartments leading to altered brain development and metabolism, and this in turn can give rise to patho-physiological and patho-developmental states (Figure 4). (4) Any situation resulting in a decreased availability of THs may potentially affect neurodevelopment. These effects depend on the dose, experimental duration, developmental period and type of biological fraction studied. These basic principles should be taken into account especially whenever free radical generation and cell death may be involved.

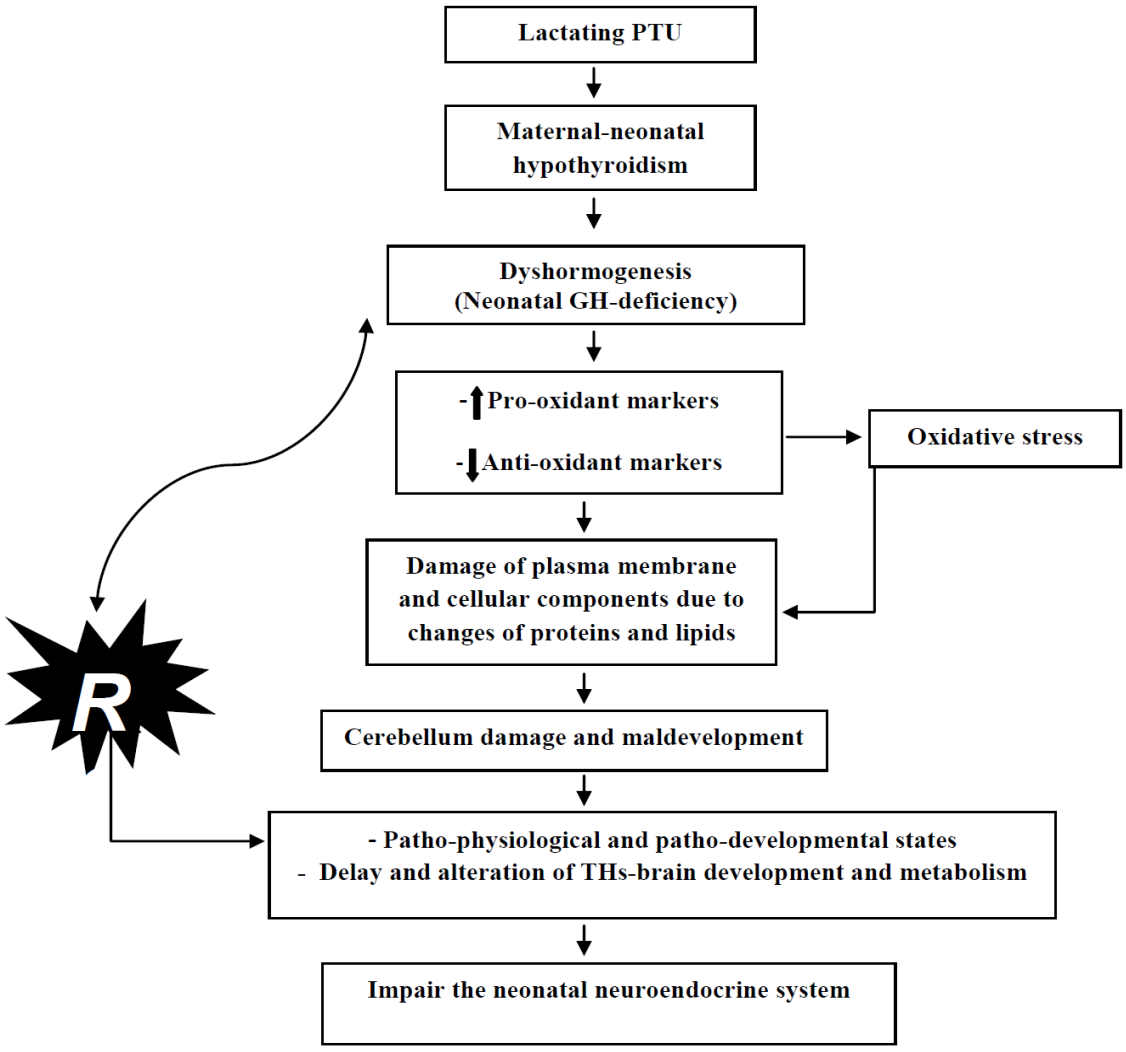

Figure 4. Schematic diagram for the toxicology of lactating PTU on the developmental neuroendocrine system, where R is radicals.

RECOMMENDATIONS AND FUTURE DIRECTIONS

- Serum TSH testing should be done in dams and their newborns during the early lactation period so that hypothyroidism can be diagnosed early and treated.
- It will certainly be of interest to explore whether the interactions between the prooxidant/antioxidant markers and the developmental neuroendocrine system (THs-brain axis) play a role in regulating signaling pathways associated with cell proliferation and cell death during the postnatal hypothyroidism.

Conflict of interest

None

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