

# THE IMMUNOHISTOCHEMICAL LOCALIZATION OF BAX IN THE BRAIN OF HYPOTHYROID NEONATE DURING MATERNAL MELATONIN INTAKE

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## ABSTRACT

**Objective:** To immunohistochemically localize Bax proteins in pyramidal cells of cerebral cortex in hypothyroid neonates and to observe the effect of melatonin on these cells.

**Study Design:** Randomized controlled trial.

**Place and duration of study:** Animal House and Anatomy Department at University of Health Sciences, Lahore.

**Material and Methods:** Nine female wistar rats were randomly divided equally into three groups, including control(A), propylthiouracyl(B), melatonin treated propylthiouracyl (C) group and allowed to conceive. Medication was given throughout pregnancy and lactation. After delivery, 10 neonatal rats from each group were sacrificed on 22nd day of life and blood samples were immediately collected from the cardiac region for evaluating serum levels of TSH. The freshly extracted brains were immediately immersed in neutral buffered formalin for 3 weeks and later further processed. 3 µm thick sections were sliced from cerebrum, deparaffinized and rehydrated, then washed in phosphate buffered saline and subsequently treated with primary and secondary antibodies.

**Results:** PTU group had significant number of pyramidal neurons in cerebral cortex showing signs of apoptosis, whereas melatonin treated group showed reversal of these signs. Moreover, on serum analysis, the pups of dams taking PTU were severely hypothyroid whereas melatonin treated pups showed significant restoration of serum TSH levels.

**Conclusion:** Melatonin preserves the structure and function of pyramidal neurons of cerebral cortex of neonates if ingested by hypothyroid mothers during gestation and lactation.

**KEY WORDS:** Melatonin, Propylthiouracyl, hypothyroidism, thyroid stimulating hormone, anti-bax antibodies.

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## INTRODUCTION

Maternal hypothyroidism is significantly damaging for development of the fetus throughout

gestation [1].

Newborns which remain undiagnosed after birth or are treated incompletely for hypothyroidism

are more liable to brain damage and learning disabilities [2].

During the first trimester, thyroid hormones (THs) are supplied exclusively by the mother, whereas during and after the second trimester, THs are supplied primarily by the mother and also by the fetus [3].

Cellular actions of THs may be initiated within the cell nucleus, at the plasma membrane, in cytoplasm, and at the mitochondrial level in every cell of the body [4]. Migration of neurons in the cerebral cortex is very sensitive to THs, and even a negligible deficiency can lead to serious consequences, including defect in migration of neurons [5]. It has been stated that hypothyroidism causes lethargy, hyporeflexia and poor motor coordination [5], and these features point to degeneration of upper motor neurons.

The role of apoptosis during neurogenesis is important in regulating the constancy of cells in developing brain. It has been observed that TH deficiency leads to extensive apoptosis during neurogenesis [6]. Bax and Bcl-2 are members of a family of intracellular, membrane-associated proteins that regulate programmed cell death [7]. It is stated that when the levels of Bax proteins are high in the cytosol of neurons, programmed cell death is enhanced and inhibition of apoptosis by Bcl-2 is suppressed [7].

Congenital hypothyroidism increases not only the severity but also the time interval of apoptosis by down-regulating anti-apoptotic gene Bcl-2 and sustaining a high level of the pro-apoptotic gene Bax<sup>6</sup>. As neurons have a lengthy lifespan, the brain mitochondria are comparatively resistant to the generation of reactive oxygen species and to the oxidative stress [8]. However, once generated, the oxidative stress provokes considerably more cell damage in brain than in other tissues. A vicious cycle thus results with the final consequence being cell death by necrosis or apoptosis [8].

Melatonin is recognized as a forager of free radicals, as a highly potent antioxidant and antiapoptotic agent synthesized not only in the pineal gland but also in peripheral tissues [9].

It has diversity of important physiological functions, including regulation of circadian

rhythm, apart from visual, reproductive, cerebrovascular, neuroendocrine, and neuroimmunological actions [10].

Neuroprotective effect of melatonin in the fetal and neonatal brain has been reported in animal studies [11]. Melatonin also protects brain in many diseases of the central nervous system including Parkinson's disease, Alzheimer's disease, and ischemic brain injury [12]. Lately, it has been stated that melatonin also effects growth and variation of neural stem cells [13]. Melatonin has both hydrophilic and lipophilic properties and this is the reason why it crosses all biologic barriers easily. It has free access to all compartments of the cell, and can be especially concentrated in the nucleus and mitochondria [14].

There is evidence suggesting that oxidative stress in neurons can be minimized by melatonin [15]. However, it is unknown whether melatonin can compensate for mitochondrial DNA damage induced in the fetal brain by reduction of maternal THs level and whether it has any effect on gene expression levels altered by low levels of maternal THs.

THs are known to be necessary for the maintenance of ideal intellectual capability in adults [5]. The relationship between balanced performance and thyroid status has been demonstrated by experimental, clinical, and epidemiological studies [5]. Many experiments have been conducted highlighting the effect of hypothyroidism on hippocampus and cerebellum [16,17], but little information is available on the effect of low TH levels on pyramidal cells of cerebral cortex during intrauterine life.

The cerebral cortex is the brain's outer layer of neural tissue in humans and other mammals [18]. Mammalian brain consists of a six-layered cerebral cortex which are numbered with Roman numerals from superficial to deep. The pyramidal cells are the main cell type within layers III and V. The present study was undertaken to immunohistochemically localize Bax proteins in pyramidal cells of cerebral cortex in the brain of neonates born to hypothyroid dams.

## **MATERIALS AND METHODS**

This experiment was conducted in the Animal House and Department of Anatomy of

University of Health Sciences (UHS), Lahore. All animal related procedures were performed and approved in accordance with the recommendations and guidelines of the committee on the Ethics of Animal Experiments for medical research at UHS.

Nine female wistar rats in good health, 12-16 weeks old & weighing between 150-250 grams, were randomly divided equally into three groups a week before mating. After a week, in individual cages, three female rats were allowed to mate with one male rat.

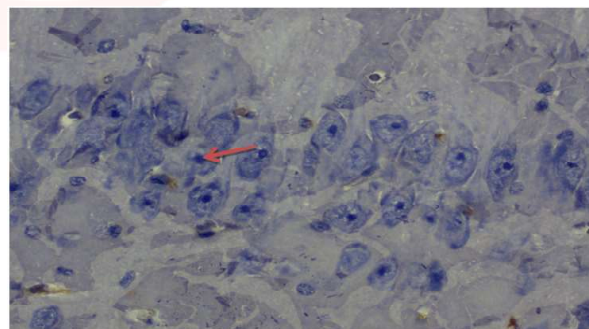
Group A served as control group and received plain drinking water and chow. Group B received 15mg/kg/day of propylthiouracyl [19] (PTU) orally a week before mating and throughout the period of gestation and weaning upto 22nd day after delivery. The purpose of administering PTU was to induce hypothyroidism in the female rats. Group C (PTU and melatonin treated group) was given 10mg of melatonin/100ml of drinking water one week before mating and PTU administered in the same dose as group B, from first day of mating daily till 22nd day of weaning.

The dams in all the groups were given medication throughout the period of gestation and weaning, and the pups were allowed free access to maternal milk after delivery. A total of thirty neonatal rats, 10 from each group, were used in the study. Only those pups from group B were included in the study whose maternal serum levels of thyroid stimulating hormone (TSH) were increased by PTU and subclinically labelled hypothyroid. All the pups were sacrificed on 22nd day of life. Blood samples were immediately collected from the cardiac region for evaluating serum levels of TSH using Elisa kit, as it is the most reliable test for diagnosing primary hypothyroidism. Immunohistochemical analysis of brain tissue was carried out only after confirming the values of serum TSH of all experimental groups. For immunohistochemical staining, 3  $\mu$ m thick sections were cut and mounted on poly-L-lysine coated slides, which were then incubated in oven at 60°C for an hour. The sections were later rehydrated by passing them through a descending alcohol series followed by a wash in running tap water. For antigen retrieval, the coplin jars were filled with sufficient retrieval solution, sealed and placed

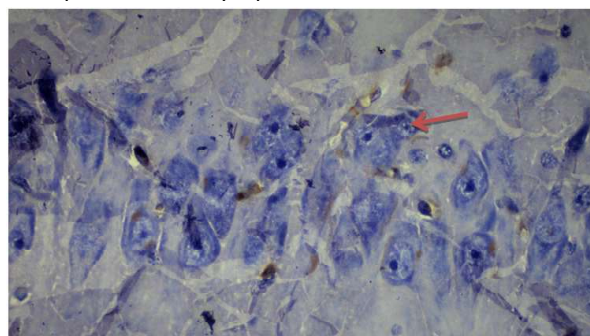
in water bath at 60°C for an hour. After cooling to room temperature, the sections were rinsed two to three times with DAKO washing buffer and two drops of hydrogen peroxide blocking solution was applied to cover the section and then incubated for 10 minutes in humidity chamber at room temperature. After washing with a wash buffer, enough diluted primary anti-bax antibody was applied to cover the section. It was once again incubated for an hour in humidity chamber at room temperature and later washed with a wash buffer. Enough HRP secondary antibody was applied to cover each section and again incubated for 20 to 30 minutes in humidity chamber at room temperature. After washing once again with wash buffer, substrate Chromogen DAB (3,3'-Diaminobenzidine) solution was applied to cover each section and incubated for two minutes and later washed with distilled water. The slides were now counter-stained with Hematoxylin and rinsed in tap water, dehydrated in ascending series of alcohol, cleared in xylene and mounted in DPX to be visualized under the light microscope.

## RESULTS

**Fig. 1: Group A:** Pyramidal cells of cerebral cortex at 40x magnification, showing normal morphology of nuclei (arrow pointing) and cell membranes. Anti-Bax antibodies are poorly localized.

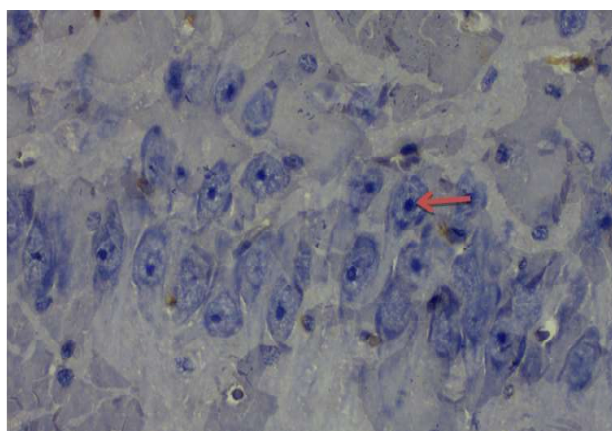


**Fig. 2: Group B:** Pyramidal cells of cerebral cortex at 40x magnification. The architecture of cell membrane and cytoplasm is distorted and their nuclei are pyknotic (arrow pointing). Anti-Bax antibodies are widespread in the cytoplasm.





**Fig. 3: Group C:** Pyramidal cells of cerebral cortex at 40x magnification. The architecture of cell membrane and cytoplasm is preserved in most of the pyramidal cells and their nuclei are of normal size (arrow pointing). Anti-Bax antibodies are poorly localized in the cytoplasm.



**Table 1:** Mean values of TSH in all the groups.

Group	TSH (ng/dl)	P value
A	9 ± 0.1	0.143
B	17 ± 0.2	0.005**
C	12 ± 0.1	0.032*

Data are expressed as mean ± S.D by using One way Anova. P value: P < 0.05. Significant \*, Highly significant\*\*

When the mean serum levels of TSH were measured, it was observed that PTU group (B) had highly significant levels of this hormone (Table-1) as compared to control(A) and melatonin treated group (C).

On immunohistochemical staining, the expression of Bax in the cytosol of hypothyroid (group B) pups was significantly more visible than that of groups A and C.

**Table 2:** Features of Pyramidal cells of cerebral cortex in all the experimental groups.

Features	Group A	Group B	Group C
Morphology of cell membrane	Well preserved	Disrupted	Preserved in most of the pyramidal cells
Size of the cell body	Large	Shrunken	Variable
Fragmentation /Chromatin condensation in the nucleus	Nucleus well preserved with no signs of apoptosis	Pyknotic nucleus with Condensed chromatin	Very few pyknotic nuclei visualized
Extent of anti-bax antibody staining	Poorly localized	Widespread	Poorly localized

## DISCUSSION

Although there are many citations available on the effect of THs on brain development, but none regarding the role of melatonin on the development of brain in hypothyroid state. Literature supports the role of melatonin in the regulation of thyroid hormone synthesis [20], but there is no information on the physiological effects of melatonin on the structure and function of thyroid gland.

In our study, there was a significant difference in serum levels of TSH between group B and C (Table- 1), signifying the effect of melatonin in regulating the activity of thyroid gland during hypothyroid state. This was similar to a study conducted by Garcia-Marin et al. in 2015 [21], who found out that melatonin has a direct impact on the functions of thyroid hormones in cultured thyrocytes.

The present work supports the role of melatonin in maintaining the integrity of motor (pyramidal) neurons of cerebral cortex during development when ingested by pregnant

hypothyroid rats. Although the animals were sacrificed on 22<sup>nd</sup> day postpartum, it still signifies the effect of melatonin in inhibiting apoptosis during intrauterine life (Fig.2).

It is an interesting fact that the pineal gland completely develops postnatally [22]. Therefore, both the embryo and the fetus are dependent on the maternal melatonin transferred through the placenta and melatonin receptors are extensive in the embryo and fetus since early intrauterine life [23]. It has also been observed that melatonin concentrations increase in maternal blood during pregnancy, reaching a maximum at term [24].

Overall, there is a lack of standard scientific evidence on the benefits of melatonin intake during pregnancy or breastfeeding, but animal studies support the use of melatonin as a neuroprotective facilitator in fetus.

Considering the important functions of the cerebrum in controlling various motor activities, its exposure to oxidants due to low levels of thyroxine may result in developmental

irregularities. However, melatonin may preserve the integrity of the mitochondria and helps to maintain neuronal function and survival. As melatonin is found in high concentrations in mitochondrial DNA, it will reverse these changes due to its antioxidant properties and preserve the integrity of cells vulnerable to injury.

## CONCLUSION

We therefore conclude that melatonin may arise as a major therapeutic drug to preserve the structure and function of motor neurons of cerebral cortex of fetus if ingested by hypothyroid mother.

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**Conflicts of Interests:** None

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