ORIGINAL ARTICLE

MELATONIN PREVENTS APOPTOSIS IN BRAINS OF NEONATES INDUCED BY MATERNAL HYPOTHYROIDISM

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Background: Loss of motor neurons may underlie some of the deficits in cognitive functions associated with maternal hypothyroidism during fetal and neonatal period. This experiment was performed to highlight the significance of melatonin intake by the mother in hypothyroid state during gestation and lactation to preserve the integrity of motor neurons in the newborns. **Methods**: Twelve female Wistar rats were divided equally into four groups, including control (A), hypothyroid (B), melatonin treated hypothyroid (C) and only melatonin treated (D) groups and allowed to conceive. For inducing hypothyroidism, Propylthiouracyl (PTU) was administered in a dose of 15mg/kg/day orally mixed with chow a week before mating and throughout the period of gestation and weaning up till 22nd day after delivery. Melatonin was given in a dose of 10mg/kg/100ml of drinking water. After delivery, 10 neonatal rats from each group were sacrificed on 22nd day of life and blood samples were immediately collected for evaluating serum levels of T3, T4 and TSH. The freshly extracted brains were sliced into two equal parts from the midline. One half was instantly immersed in ice cold phosphate buffered saline and homogenized for extraction of RNA to determine the genetic expressions of caspases 3, 8 and 9. Other half of the brain was instantly immersed in 10% formalin for 2 weeks. After processing of the brain tissue, 5 µm thick sections were sliced and transferred to albumin coated glass slides. They were later stained by Nissl staining technique, visualized and photographed under a research microscope for signs of apoptosis. The mRNA and protein levels of caspase-3, 8, and 9 were analyzed using a real-time RT-PCR. **Results**: Serum enzyme analysis showed that the pups of dams taking PTU were severely hypothyroid and melatonin treated rats showed significant restoration of serum thyroid hormone levels. Features of apoptosis and disturbed migration of cells was seen in B group as compared to C group and mRNA levels of caspase-3 and 9 were increased significantly in B group. **Conclusion:** Melatonin helps to maintain neuronal function in hypothyroid newborn rats by inhibiting apoptosis and improving survival.

Keywords: Hypothyroidism; Melatonin; Propylthiouracil; Hippocampus; Pyramidal neurons; Caspases

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INTRODUCTION

Thyroid hormones (THs) play an indispensable role in growth and metabolic homeostasis in humans as well as in animals.[1] In the last decade, there has been a lot of consideration on the effects of THs during fetal life in terms of tissue differentiation and development.[2] Among all of the systems influenced by thyroid action, the central nervous system appears to be highly sensitive during the developmental stages.[2] Maternal hypothyroidism delays maturation of neurons and also decrease their connectivity.[3] In humans, decrease or absence of THs during stages of brain maturation causes molecular changes, as well as changes in the morphology and functions of motor neurons of the cerebral cortex, hippocampus and cerebellum.[4] The hippocampus resides in the temporal lobe of the brain in vertebrates and forms an essential component of the limbic system. It is composed of dentate gyrus and Cornu Ammonis (CA). The CA is divided into different regions or subfields titled CA1, CA2, CA3, and CA4.[5] The CA3 subfield has always fascinated researchers for its definite part in progression of memory and its vulnerability to neuro-degeneration.[6] Connections of neurons in this subfield is richer than in other hippocampal regions.[6] Axons of CA3 pyramidal neurons branch profusely to synapse with adjacent neurons.[6]

Even before the commencement of thyroid functions, the only basis of fetal TH is the thyroid gland of the mother.[7] THs have been detected in the rat embryos during second week of development and in the human fetus prior to the commencement of functions of fetal thyroid gland.[8] Moreover, even when the thyroid gland functions of the fetus are initiated during mid part of gestation in humans, fetal brain still depends on maternal THs. THs are initially transferred through the placenta and later the brain-

blood barrier. This transport favors T4 at the cost of T3.[9] Actions of TH may commence within the cell nucleus, at the cell membrane, within cytoplasm or inside mitochondrion.[10] The hippocampus is a highly sensitive neural structure to the actions of THs due to its high content of thyroid receptors.[11]

Apoptosis is a dynamic process that involves active participation of phosphates, gene expressions and caspases. It occurs normally during CNS development, in neurodegenerative diseases and recently, studies have highlighted extensive apoptosis as the major cause of delayed motor functions in neonates born to hypothyroid mothers.[12] The role of apoptosis during neurogenesis is important in regulating the constancy of cells in developing brain.[13] TH deficiency leads to extensive apoptosis during neurogenesis[4]. The mechanism by which apoptosis induces cell death may comprise both caspase-dependent and caspase-independent processes.[14]

Caspases are the primary target of apoptosis. They are "Killer" molecules, which once activated, damage cytoplasmic and nuclear processes which subsequently leads to promoting cell death by apoptosis.[14] Caspases are located in the cytoplasm of all animal cells as inactive zymogens. Breakdown of these zymogens generates active enzymes to activate apoptosis.[14] Many experiments have demonstrated a key role of caspases in loss of neurons after induction of ischemia. Intrinsic pathway for activation of caspases involves mitochondria and once initiated, discharge of cytochrome C from the intermembrane space of mitochondria follows on contact of neurons to apoptosis inducing stimuli.

This experiment was performed to highlight the significance of melatonin intake by the mother in hypothyroid state during gestation and lactation to preserve the integrity of motor neurons of new born pups. We tested whether melatonin could prevent apoptosis of motor neurons of hippocampus following exposure to low levels of THs during gestation and lactation. Moreover, this experiment was conducted to histologically observe the structure and arrangement of pyramidal cells of hippocampus for signs of apoptosis and to correlate the observed findings to the genetic expressions of caspase-3, 8, and 9 from the brain tissue in all the experimental groups.

MATERIAL AND METHODS

This research was conducted in the Animal House and Department of Anatomy of University of Health Sciences (UHS), Lahore, after obtaining ethical approval from committee on the Ethics of Animal Experiments for medical research at UHS according to the recommendations and guidelines of the committee.

Twelve female wistar rats in good health, 12–16 weeks old and weighing between 210–250 grams, were divided equally into four 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 (hypothyroid group) received 15mg/kg/day of propylthiouracyl mixed with chow orally[15] a week before mating and throughout the period of gestation and weaning up till 22nd day after delivery. Group C (melatonin treated hypothyroid group) was given 10mg of melatonin/kg/100ml[16] of drinking water one week before mating and PTU administered from 1st day of pregnancy daily up till 22nd day of weaning. Group D was only given 10mg of melatonin/kg/100ml of drinking water a week before mating and throughout the period of gestation and weaning.

As the period of gestation is around 22–23 days in albino rats, and the experiment ended on 22nd postnatal day, the duration of the study was around 54–55 days. Only those pups were included in the study whose maternal serum levels of THs was affected by PTU and were clinically labelled hypothyroid. After delivery, a total of 40 neonatal rats, 10 from each group, were used in the study. 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.

Experimentally induced hypothyroidism was achieved through Propylthiouracil (PTU), which is a thiourea derivative with antithyroid properties. Administration of PTU results in decreased plasma T3 concentrations and decreased entrance of thyroxine into cells thereby decreasing TH activity. The period of weaning in laboratory rats is around 22 days. During the initial 21 days, rats spent most of their time feeding their pups which most probably resulted in transfer of PTU into the pup's serum, as indicated by their serum analysis.

Serum total T3 (TT3), total thyroxine (TT4) and TSH of pups were estimated in accordance with manufacturer's protocol using standard ELAB kits (New York, USA). The levels were measured to notice the endocrine effects of PTU on the thyroid gland of 22 days old pups and to associate the physiological state of the thyroid gland to the histological findings in the hippocampus.

Processing of brain tissue was done as per following: After retracting the scalp, the brain was scooped out from the skull with the help of a spatula and the meninges were cut with fine scissors. One half of the freshly extracted brain was immediately immersed in 10% formalin for a week, after which further three sections of the fixed brain tissue were done for better

penetration of the fixative and left immersed in it for another week.

Two days prior to the performance of Nissl staining, the brain tissue was processed with the help of a tissue processor. The automatic tissue processor was used for tissue processing for a span of 18 hours. Tissue was dehydrated by passing through ascending grades of alcohol using 50%, 70%, 80%, 95% and absolute alcohol. Subsequently, the tissue pieces were cleared in xylene and infiltrated with molten paraffin at melting point of 58 ^{o}C. Tissues were then embedded in paraffin wax and 5 µm thick sections were sliced with the help of rotatory microtome, which were then immediately transferred to water bath kept at the temperature of 45 °C. Sections were then shifted to the surface of clean albumin coated glass slides and labeled with lead pencil. Excess water on the slides was drained off and these were then dried on the slide warmer. Nissl staining of Hippocampus for histological study was carried out as per the following: Histological study involved Nissl staining of the Hippocampus.[17] The tissue slides were loaded into holders and immersed in sequence into wells as; xylene for 5 minutes, 95% alcohol for 3 minutes, 70% alcohol for 3 minutes, deionized distilled water for 3 minutes, Cresyl Violet stain for 8–14 minutes at 60 ºC in oven, after which it was again immersed into distilled water for 3 minutes, 70% Alcohol for 3 minutes, 95% Alcohol for 2 minutes, 100% Alcohol for 1–2 seconds and then finally immersed into xylene for 5 minutes. The slides were then examined, specifically in CA3 region of the hippocampus, under the light microscope. Photomicrographs of the observations were taken at 40x magnification, using an Olympus Research Microscope (model BX51).

RNA was extracted under sterile conditions in cold environment. Isolation of RNA was done with the help of RNA isolation kit (FavorPrep Tissue Total RNA Mini Kit, Catalog #FATRK001, Taiwan).The remaining half of the fresh brain tissue was instantly immersed in ice cold phosphate buffered saline and homogenized in a dounce homogenizer after adding 1ml of isolation buffer to the tissue. The homogenate was centrifuged at 1,000xg for 10 minutes at 4 ºC. The supernatant was collected and further centrifuged at 12,000xg for 15 minutes at 4ºC. The steps and contents of the kit were closely followed according to the protocol. Then 1ml of isolation buffer and 10µl of protease inhibitor cocktail was added to the pellet and it was once again centrifuged at 12,000xg for 15 minutes at 4 ºC, frozen at -80 ºC until use.

To pinpoint a possible molecular source for the TH dependent change in apoptotis, expression of caspase 3, 8 and 9 at mRNA and protein levels were examined on 22nd day postnatally. The primers were designed using Gene Bank sequences for caspase 3, 8, and 9 (Table-1). RNA extracted from brain tissue was reverse-transcribed to form complementary DNA by using the thermocycler. Primer annealing temperatures were optimized before use (Table-1). Conventional PCR was performed under the following cycling conditions: denaturation at 95°C, annealing at 56 °C and extension at 72°C for 40 cycles.

All PCR reactions were performed in a 11 µl mixture containing 6 µl Syber Green PCR Master Mix, 1 µl sample complementary DNA, 0.5 µl forward primer, 0.5 µl reverse primer, and 3 µl RNAse free water. A negative control consisting of water was included with each reaction set. These PCR products were analyzed in agarose electrophoretic gels. After staining with ethidium bromide, UV light gel images were captured and visualized on the computer screen.

Changes in mRNA content, activity for caspase-3, 8 and 9 were analyzed using gel electrophoresis and their levels measured with a 50bp ladder. Data was analyzed using SPSS 20.0. One-way ANOVA was used for significant testing.

RESULTS

When the mean serum levels of TSH were measured, it was observed that PTU group (B) had significantly raised levels of this hormone (Table-2 A and B) as compared to the control (A) and melatonin treated group (C). However, no significant difference was observed between groups in T4 and T3 serum levels (Table 2- A).

No features of apoptosis were seen in control group A and the shape and size of the pyramidal cells in CA3 region of the hippocampus was overall unaltered (Figure-1). The pyramidal cells in CA3 region of the hippocampus were not unusual in number and were orderly organized (Figure-1). Apoptotis was confirmed in group B based on the characteristic morphological features such as overall shrinkage of the cell, nuclear fragmentation and chromatin condensation (Figure-3). Reduction in number of pyramidal neurons and misplacement of cells in CA3 region of hippocampus was also observed (Figure-2). Condensation of the nucleus and cytoplasm, which are important features of apoptosis, could not be clearly visualized. RT PCR using rat specific primers for caspase 3, 8 and 9 was performed. Total cDNA was then amplified with rat caspase-specific primers based on the available rat cDNA sequence (Table-1).

The levels of initiator caspase 9 were significantly high in B group whereas it was unremarkable in other groups. Likewise, caspase 3 levels were also raised in group B as compared to group C. Caspase 8 levels were not raised in any of the experimental groups.

Table-1: Gene sequences of caspase 3, 8, 9.

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
CASP3	GGCCGACTTCCTGTATGCTTAC	GACCCGTCCCTTGAATTTCTC
CASP8	GATTACGAACGATCAAGCACAGA	ATGGTCACCTCATCCAAAACAGA
CASP9	TCTGGCAGAGCTCATGATGTCT	GGTGTATGCCATATCTGCATGTCT

Table-2: Mean values of TSH, T3 and T4 in all the groups

Groups	TSH (ng/dl)	T3 (ng/dl)	T4 (ng/dl)
A	10±2.6	35.7±2.5	373±4
B	21±4.5*	32.7±1.5	33.3±4
C	12.67±2	34.7±3.8	33.3±1.5
D	12±1	33±3	32±2

Data are expressed as mean±S.D by using One-way Anova. p <0.05 is significant*

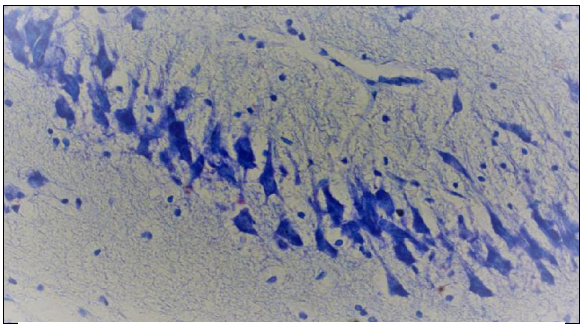

Figure-1: Photomicrograph showing pyramidal cells of hippocampus in control group showing normal morphology and arrangement of cells in CA3 region.

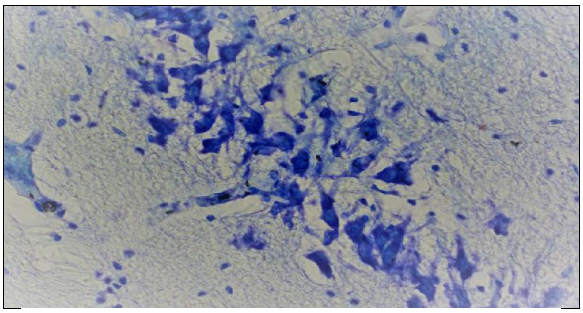

Figure-2: Photomicrograph showing pyramidal cells of hippocampus in group B. The architecture and arrangement of cells is disturbed.

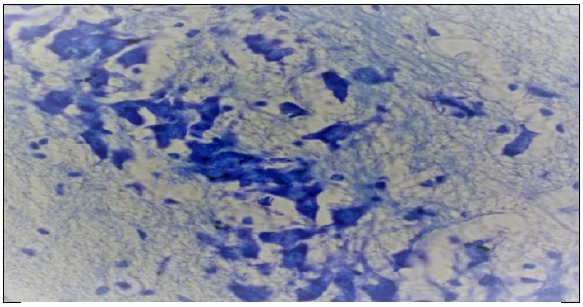

Figure-3: Photomicrograph showing pyramidal cells of hippocampus in group B showing signs of apoptosis, including irregular cell boundaries and fragmentation of nuclei. Reduction of pyramidalneurons and their disturbed migration.

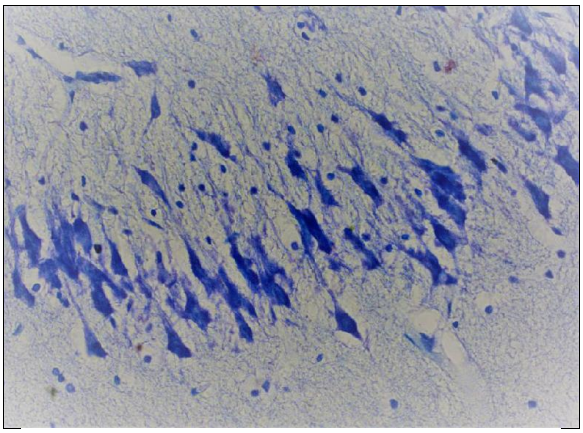

Figure-4: Photomicrograph showing pyramidal cells of hippocampus in group C. The overall architecture and arrangement of cells is preserved.

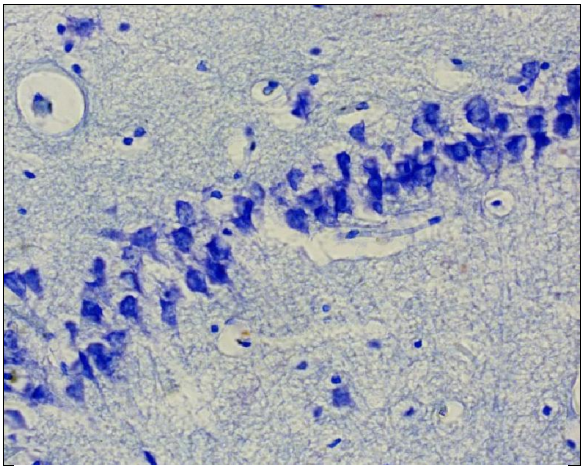

Figure-5: Photomicrograph of pyramidal cells of CA3 region of hippocampus in group D showing normal morphology and arrangement of cells. The outlines of the cell and nuclear membrane are normal.

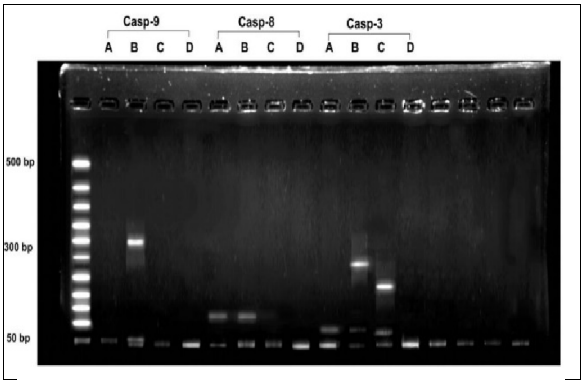

Figure-6: Gel expressions of caspase 9, 8, 3 in all groups.

DISCUSSION

The relationship between maternal hypothyroidism and fetal neuronal activity is complex. It is stated that even a slight shift in the serum levels of THs leads to disturbed mitochondrial functions.[18] A substantial increase in the serum levels of TSH in group B was observed (Table-2A) when compared to other groups, but T3 and T4 levels showed no remarkable change amongst any of the groups (Table-2). This indicated subclinical hypothyroidism in B group and it has been stated that even a slight shift in serum TSH levels can result in serious consequences.[19]

It is a well-documented fact that hypothyroidism causes severe structural irregularities in the developing brain as a whole.[4,19] The size and number of neurons, especially pyramidal neurons in the neocortex and hippocampus are reduced.[20] Similar was the case in our experiment where the number of pyramidal neurons in CA3 region of the hippocampus was reduced in hypothyroid (B) group (Figure-2). It has been observed by many researchers that THs strongly influences migration of cells in the cerebral cortex, hippocampus and cerebellum and even slight shift in their serum content can result in disturbed migration.[21] For example, in an experiment conducted by Zoeller in 2003[22], short-lived maternal hypothyroidism in pregnant rats at embryonic days 12–15 caused remarkable misplacement of cells in the cerebral cortex and hippocampus when analyzed at 40 days of age. It was observed that even temporary hypothyroxinemia in mother resulted in permanent change on the anatomy of the cortex and hippocampus of offspring. Likewise, in our study, disturbed migration of pyramidal cells was witnessed by haphazard arrangement of pyramidal cells in CA3 region of hippocampus (Figure-2), when compared with group A (Figure-1) and D group (Figure-5).

Loss of mitochondrial barrier function in intrinsic pathway of apoptosis is a prerequisite for caspase 9 to reach its substrates, which is an initiator caspase responsible for initiating the intrinsic apoptotic pathway.[23] Caspase-9, which forms part of the pathway signaling through the mitochondria, showed an increase in expression in group B in the present study, when compared to other groups (Table-3). The initiator caspase 9 then activates the executioner caspases, such as caspase 3, which bring about the apoptotic destruction of the cell. Likewise, expressions of caspase 3 were significantly high as compared to 8 and 9 in group B. Group C showed a substantial reduction in the expressions of caspase 9 and 3, highlighting the anti-apoptotic effect of melatonin (Figure-6). In contrast, caspase 8, which forms part of the extrinsic apoptotic pathway, showed no remarkable change in expressions of any of the three primers (Figure-6).

Histological study showed shrunken, pyknotic neurons in CA3 region of hippocampus in B group (Fugre-3). This change was most probably due to apoptosis and increased oxidative stress may have been involved for these changes.[24] On the other hand, in C group, the histological picture was not that of apoptosis, as the architecture of the pyramidal neurons was preserved significantly (Fugre-4). In group D, the anatomy of pyramidal neurons was well intact (Fugre-5) and their arrangement was also in order. This finding was significant as it indicated melatonin as an anti-apoptotic agent.

There is a strong indication that melatonin can significantly reduce mtDNA damage.[25] Thorough research has specified melatonin's beneficial effects in experimental models of neurodegeneration. There is increasing evidence that its anti-apoptotic effects play an important role in preventing neurons from degenerating[26] and similar results of its use were observed in our study. The highest levels of melatonin are found in the mitochondria in all living organisms, and this maybe the reason why it has a direct effect on this organelle, as it can gather in many folds greater concentration within mitochondria and protect it from oxidative damage.[27] As it is lipophilic, it accumulates in the inner mitochondrial membrane, close to the electron transport chain.[28] Melatonin promotes mitochondrial homeostasis and inhibits mitochondrial cell death pathways.[27,28]

Although hypothyroidism is not a neurodegenerative disease, but low levels of maternal THs causes loss of neurons via extensive apoptosis during intrauterine life. Experiments support the role of melatonin as an anti-apoptotic agent in neurodegenerative diseases, but no study has been conducted so far on melatonin in preserving the structure of pyramidal neurons of hippocampus during maternal hypothyroid state.

CONCLUSION

There is lack of evidence to link fetal neuronal damage during maternal hypothyroidism to the inhibition of apoptosis by maternal intake of melatonin. By inhibiting apoptosis, melatonin helps to maintain neuronal function and survival. It is assumed that these findings will have significant applications.

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Competing Interests

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

MH: Design, Statistical analysis, editing. SC & SS: Data collection, manuscript writing. KPL: Reviewed and approved the manuscript.

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