Melatonin Protects the Purkinje Neurons of Cerebellum of Neonates from Apoptosis Induced by Maternal Hypothyroidism in Wistar Rats

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ABSTRACT

This study was designed to evaluate the effect of melatonin in preventing apoptosis of Purkinje neurons in 22 days old pups born to hypothyroid Wistar albino rats. Sixteen mother rats were equally divided into 4 groups (A, B, C, D). Their medication started a week earlier before they were allowed to conceive and continued during gestation and breastfeeding till weaning of offspring. Group A was the control. Group B was given propylthiouracil (PTU) to inhibit their thyroid gland activity which resulted in increased serum TSH and reduced T4 levels. Group C was administered both PTU and melatonin, whereas group D was administered melatonin only. In melatonin treated hypothyroid rats (group C), significant findings were observed. This study highlighted the potential protective mechanisms of melatonin in Purkinje cells of pups born to hypothyroid dams. Melatonin restored the serum levels of TSH and T4 and down-regulated the gene expressions of Bax along with caspases 3 and 9 in Purkinje cells of pups. Thus melatonin suppressed the neuronal apoptosis induced by maternal hypothyroidism. Considering the limited remedies to effectively treat hypothyroidism associated with neonatal brain damage, melatonin may provide an alternative method for this disorder.

Keywords: Hypothyroidism, propylthiouracyl, melatonin, cerebellum, purkinje neurons, apoptosis, thyroid hormones.

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INTRODUCTION

In humans, maternal hypothyroidism harms the development of several tissues and organs of the fetus throughout pregnancy (Moog et al., 2015). The fetal brain is the most vulnerable organ to suffer from the imbalance of thyroid hormones (THs) during the first trimester in which the maternal thyroid gland is the only source of this hormone (Bernal et al., 2003). TH receptors are found in the highest concentration in developing neurons of fetal brain. They are detectable by 10th week of gestation in humans and 2nd week of gestation in rodents (Choksi et al., 2003).

Considerable actions of THs on central nervous system development occur during the first weeks of pregnancy. That is the time when the levels of THs are lower in the developing fetus as compared to those in the mother both in rodents and humans (Stoica et al., 2007). The precise mechanism by which thyroid hormone induces its effects in developing the brain is poorly understood. It is assumed that these effects are facilitated via nuclear receptors (Schwartz et al., 1997). Even a temporary deficiency or increase of maternal thyroid hormones during pregnancy can have damaging results on the gross structure of the brain in the offspring (Stenzel and Huttner, 2013). It is a well-documented fact that hypothyroidism causes severe morphological abnormalities in the entire developing brain (Singh et al., 2003).

The cerebellum is a very important part of the central nervous system at the lower back of the brain that plays an essential role in motor coordination. It also plays a central role in regulating many activities of the cerebral cortex (Hull and Regehr, 2012). The cerebellar cortex

consists of 3 layers: the molecular layer, Purkinje layer and granular layer. Purkinje cells, found within the Purkinje layer, are some of the largest motor neurons in the human brain, with a complex and detailed dendritic tree, containing a large number of dendritic spines (Snell, 2010). They are found in a single layer and play a substantial role in the developing cerebellum. The position and number of the Purkinje neurons are important for forming synaptic connections with adjacent neurons in the molecular layer and granular layer (Li et al., 2010).

The major defect in the cerebellum of hypothyroid rats involves the Purkinje cells (Heuer and Mason, 2003). The location and number of the Purkinje cells are not affected, but the development of their dendritic tree is slower (Ahmed et al., 2008). This results in impairment of synapsis of Purkinje neurons with the granule cell neurons causing weakened neuronal connectivity. These abnormalities can be reversed if treatment with TH is given before the critical period of cerebellar development, i.e., before twenty-one days of age in the rat (Bernal., 2007).

The pineal gland is a part of the diencephalon which is located infero-posterior to the thalamus (Patton, 2018). It consists of cells called pinealocytes which secrete the hormone melatonin. Melatonin is derived from serotonin in reaction to low light levels. In mammals, the main melatonin source is the pineal gland. It is found in all organisms, ranging from unicellular to vertebrates (Pévet, 2002) and is a well-known free radical scavenger, antioxidant and antiapoptotic agent (Tan et al, 2015).

Melatonin is very significant for the brain of the developing fetus as it stimulates a number of antioxidant enzymes (Ding et al., 2014). It can preserve the cell membrane, organelles and combat free radical damage (Reiter et al., 2018). Various animal studies have highlighted the neuroprotective effect of melatonin in the fetal brain (Alonso-Alconada, 2013). It is shown that melatonin increases the activity of glutathione peroxidase in rat brain (Şenol and Nazıroğlu, 2014). It exerts neuroprotective effects in Parkinson's disease, Alzheimer's disease, and ischemic brain injury (Yu et al., 2017). It easily crosses blood-brain barrier and can be found in large concentrations in the brain after exogenous administration (Dubocovich, 2010). Melatonin also stimulates growth and differentiation of neural stem cells (Moriya, 2007).

There remains a gap in knowledge concerning the role of melatonin on Purkinje neurons during hypothyroidism induced neuronal apoptosis. 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 fetal and neonatal neurons during maternal hypothyroid state. Moreover, there is a lot of literature available on the effect of hypothyroidism on the morphology of Purkinje neurons, but those studies are limited to its effects on the dendritic tree and myelination process of axons. This study has highlighted the structural and functional alterations of Purkinje neurons in neonates leading to apoptosis during maternal hypothyroidism. Moreover, it has emphasized the importance of melatonin intake by the mother in preventing extensive apoptosis of neurons.

MATERIALS and METHODS

Experimental Animals

The animals were purchased from the University of Veterinary Animal Sciences, Lahore, Pakistan. The animal study was performed in the Experimental Research Lab and Department of Anatomy of the University of Health Sciences (UHS), Lahore. All animal-related procedures were conducted in accordance with the guidelines of the Ethical Review Committee for medical research at UHS and appropriate measures were taken to reduce the pain of experimental animals. The ARRIVE guidelines for experimental research on animals were followed. The approved protocol number for this study is UHS/ERC/126-17/19. The animals were housed in a temperature-controlled room with 22 ± 1ºC and with the light/ dark cycle of 12 h /12 h (light on at 8:00 and off at 20:00). The animals were allowed to access food and water at libitum. The study was started after one week of acclimatization of the animals.

Experimental Design

Sixteen healthy female Wistar rats, 12-16 weeks old, in the weight range of 200-220 g, were divided equally and randomly into four groups. Group A served as control and received plain drinking water and chow. Group B received 15mg/kg/day of propylthiouracyl (PTU) orally mixed with chow (Alkalby, 2013). Group C received PTU in the same dose along with melatonin (10mg of melatonin/kg/day in drinking water (Hidayat, 2012). Group D was given 10mg of melatonin/kg/day only in drinking water. After one week of treatment, the female rats were allowed to mate with males in order to conceive. The treatment of dams continued throughout the period of gestation and breastfeeding till weaning of offspring. During the period of the experiment, the serum levels of TSH, T3 and T4 of the dams were measured weekly and it was made sure that the TSH levels of group B mother rats were significantly affected by PTU in order to label them hypothyroid. After delivery, all the pups were allowed free access to maternal milk as that was used as the only source of transfer of medication to the growing pups. They were not given any medication directly. A total of 40 pups, 10 from each group, were used in the study and they were sacrificed on the 22nd day of their life.

Analysis of Serum TSH, T3 and T4 of pups

Blood samples were collected from the cardiac region for

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

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
CASP 3	GGCCGACTTCCTGTATGCTTAC	GACCCGTCCCTTGAATTTCTC
CASP 8	GATTACGAACGATCAAGCACAGA	ATGGTCACCTCATCCAAAACAGA
CASP 9	TCTGGCAGAGCTCATGATGTCT	GGTGTATGCCATATCTGCATGTCT

an immediate evaluation of serum levels of TSH, T3 and T4 using Elisa kit following the instructions of the manufacturer (Abnova Catalog # KA2336 for TSH, Catalog # : KA0925 for T3, Catalog # : KA2335 for T4).

Isolation and extraction of RNA

Neonatal brain was excised from the skull. One half of the freshly extracted brain was instantly immersed in ice-cold phosphate-buffered saline and homogenized in a dounce homogenizer after adding 1ml of isolation buffer to the tissue containing 225 mM sucrose, 75 mM mannitol, 1 mM EDETA and 5 mM Hepes at pH 7.4. The remaining half of the brain was used for immunohistochemical study.
The whole process of RNA extraction was performed under cold environment and sterile conditions. Extraction and isolation of RNA were done with the help of RNA isolation kit (FavorPrep Tissue Total RNA Mini Kit, Catalog #FATRK001, Taiwan). The isolation procedure strictly followed the protocol provided by the manufacturer. A total of 0.3-0.4 g of brain tissue was extracted and centrifuged at 1,000xg for 10 min at 4ºC. The pellet was discarded and the supernatant was collected and further centrifuged at 12,000xg for 15 min at 4ºC. This time, the pellet was preserved and the supernatant discarded. Then 0.5ml of isolation buffer and 5μl of protease inhibitor cocktail were added to each pellet and centrifuged at 12,000xg for another 15 min at 4ºC. The precipitants obtained were the isolated RNAs. After adding 40-100 μl of RNA free distilled water to it, the RNAs were frozen at -80 ºC until use. The primers were designed using Gene Bank sequences for caspase 3, 8, and 9 (Table 1).

PCR analysis

RNA extracted from brain tissue was reverse-transcribed to form cDNA by using the thermocycler. Primer annealing temperatures were optimized before use. Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) was used 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 11μl of mixture containing 6 μl of SYBR Green PCR Master Mix, 1 μl of sample cDNA, 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. The threshold cycle (Ct) for each well was calculated using the instrument's software and the melting-curve program was immediately run after the cycling program. Data analysis was done by the ΔΔCt method.

Immunohistochemical analysis of apoptosis of Purkinje cells of cerebellum

The 3 μm thick sections of hippocampus mounted on slides were incubated in an oven at 60ºC for an hour and then, processed with a series of descending concentrations of alcohol followed by a wash in running tap water. For antigen retrieval, the slides were immersed in this solution and kept in a water bath at 60ºC for an hour. After cooling to room temperature, the sections were rinsed three times with DAKO washing buffer and then, two drops of hydrogen peroxide blocking solution were applied to cover the section and incubated for 10 min in a humidity chamber at room temperature. After washing with wash buffer, the primary anti-bax antibody (Mouse Monoclonal to BAX purchased from St John's Laboratory, USA) in a dilution ratio of 1:100 was added to samples and incubated for another hour. After washing, the HRP secondary antibody (ready to use) was added to the samples for half an hour and washed with Phosphate Buffered Saline. After washing, a chromogen named DAB (Di-aminobenzidine) was added to the sample for two min and then rinsed with distilled water. The slides were then counter-stained with Hematoxylin and rinsed in tap water, dehydrated in series of ascending concentrations of alcohol, cleared in xylene and mounted in DPX. The Purkinje neurons in the cerebellar cortex were analyzed under a light microscope. Briefly, a total of 3 sections from each slide (15 sections/ animal) were photographed.
The Purkinje cell count was carried out by over the ocular graticule with a calibration factor of 40 μm. The Purkinje neurons inside the squares of the grid were recorded except the cells lying on the lower and left edge of the grid. The cells were counted randomly in selected three areas in each section at the magnification of 63X; and the mean was calculated using SPSS 20. The parameters used to label the cells as apoptotic were the size of the cell body, fragmentation of the nucleus, the extent of Bax staining of the cell nucleus or cytoplasm. Mean Purkinje cell count was expressed as the number of Purkinje cells per mm^2 for both normal and apoptotic cells.

Statistical analysis

Data were presented as mean ± SD. One-way analysis of variance (ANOVA) was used for normality analysis
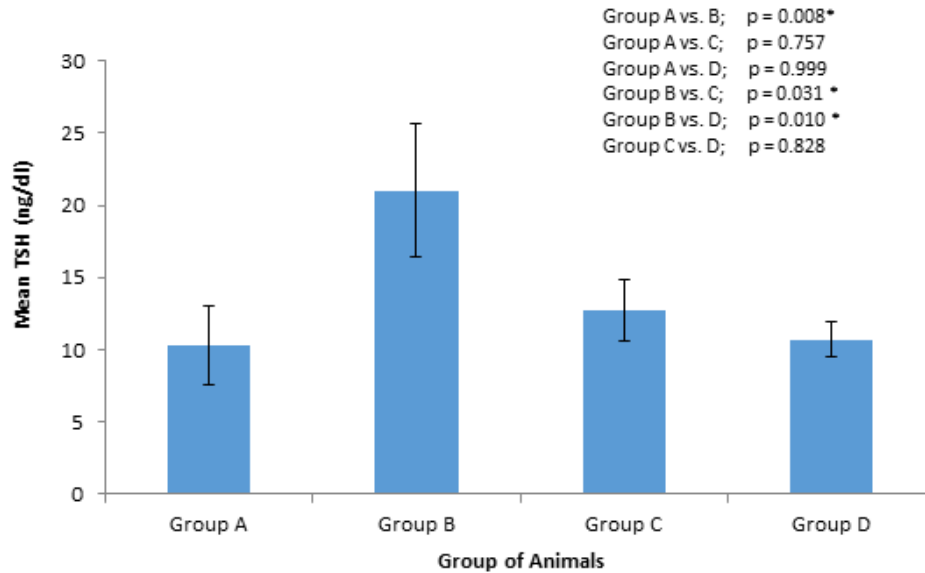
Figure 1. Effects of PTU and melatonin on the serum levels of TSH of pups.
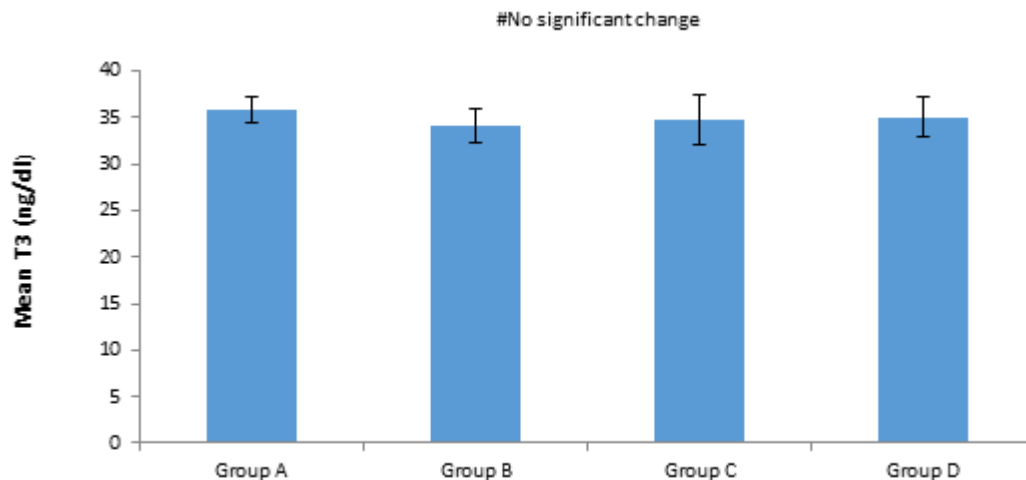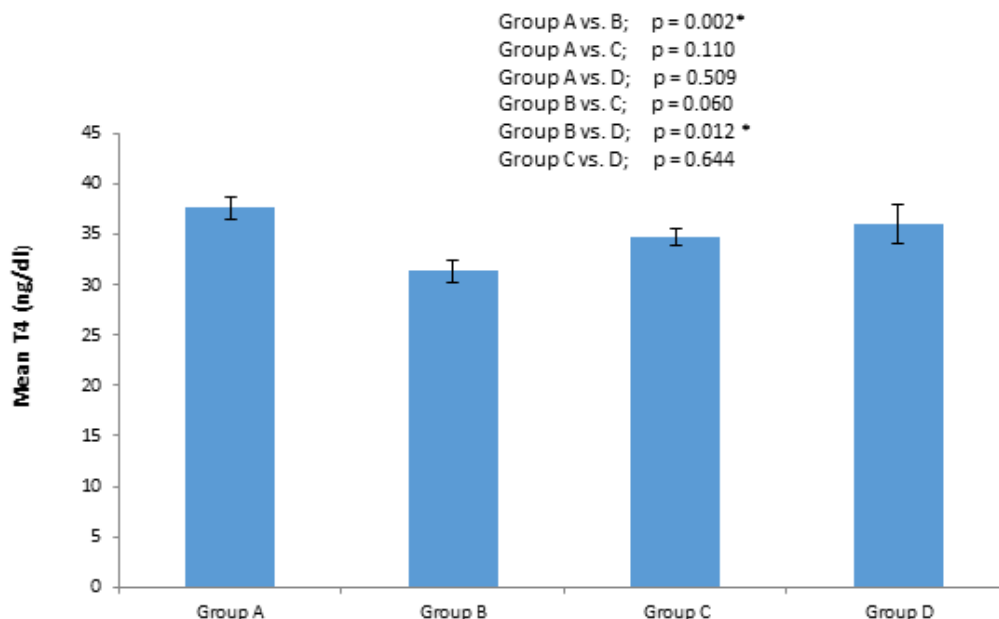

Figure 2. Effect of PTU and melatonin on the serum levels of T3 of pups.

among groups, followed by Tukey's post-hoc analysis to obtain the statistical significant difference of various quantitative changes between the groups. $p < 0.05$ was considered to be statistically significant. All calculations were done by utilizing computer software SPSS version 20.

RESULTS

The data were expressed as mean ± SD (n = 10). Group A: control, Group B: treated with PTU, Group C: treated with PTU and melatonin, Group D: melatonin treated alone. *indicated statistical significance vs the relative group. After applying Post Hoc Tukey's Test, the P-value was highly significant in B group (P< 0.001) as compared to other groups.

The result showed that PTU treatment significantly increased serum levels of TSH ($P < 0.05$) compared to control (Figure 1). Melatonin alone did not modify the TSH level. However, in the animals co-treated with PTU and melatonin, their pups TSH levels showed insignificant changes in comparison with the control group. This indicated that melatonin significantly suppressed the rise of TSH induced by PTU (Figure 1). There were no significant alterations observed in serum total T3 levels among this group (Figure 2). In contrast, serum total T4 level was significantly lower in pups of PTU treated dams than that pups of control dams (Figure 3). Melatonin alone did not alter the T4 level. However, in the female rats co-treated with PTU and melatonin, their neonates T4 level was significantly elevated compared to the only PTU treated rats ($p < 0.05$) (Figure 3).

Group A vs. B;   p = 0.002*
Group A vs. C;   p = 0.110
Group A vs. D;   p = 0.509
Group B vs. C;   p = 0.060
Group B vs. D;   p = 0.012 *
Group C vs. D;   p = 0.644

Figure 3. Effect of PTU and melatonin on the serum levels of T4 of pups.

The levels of T4 were significantly decreased in group B as compared to other groups.* $p < 0.05$ vs other groups.

Immunohistochemical findings of Purkinje cells of cerebellum

The expression of Bax, an index of apoptosis, in the nucleus and cytoplasm of Purkinje cells was significantly upregulated in PTU group B compared to control group A (Figure 4B). The nucleus was shrunken and pyknotic (Figure 4C). Melatonin treatment significantly reduced the neuronal apoptosis induced by PTU (Figure 4D). Melatonin alone preserved the neurons as they were in the control group (Figure 4E). The statistical analysis of the normal neurons and apoptotic neurons in this area are illustrated in Figure 5.

Figure 4 shows the Immunohistochemical staining of Bax (100x magnification) of Pyramidal cells of cerebellar cortex. The square in topmost panel A indicates the layers of cerebellar cortex with arrows pointing at the Purkinje cells (10x). The panel B, C, D and E are the magnified Purkinje cells in each treatment group. B: control, C: PTU, D: PTU plus melatonin, E: melatonin alone. Arrows in C and D point to the pyknotic nuclei with Bax staining. The damage in C was significantly intense compared to D. The arrows in B and E point to the normal nuclei whereas those in C and D point to the apoptotic nuclei.

The normal Purkinje cell count varied from 11 – 13 cells / unit area in all groups whereas the count of apoptotic cells was increased significantly ($P < 0.05$) in B group (mean 3-4 cells per unit area) as compared to C group (mean 2-3 cells per unit area), D group (mean 0-1cells per unit area) and A group (mean 1-2 cells per unit area).

Gene expression analysis of caspase 3, 8 and 9 from brain tissue of all experimental groups

The data were expressed as mean ± SD (n=10). The numbers in the Y-axis were the altered folds of the gene expression after normalizing the values with a housekeeping gene β-Actin mRNA.

The level of initiator caspase 9 was significantly high in group B ($p < 0.001$) whereas it was unremarkable in other groups (Figure 6). Caspase 8 levels were not raised in any of the experimental groups (Figure 6).

DISCUSSION

This experiment has highlighted the potential protective mechanism of melatonin on Purkinje cells of pups born to hypothyroid dams. 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 (Moon et al., 2016). Since melatonin is a versatile neuroprotector, it is hypothesized that melatonin may provide protective effects on hypothyroid related brain damage in neonates, especially, that melatonin has been reported to regulate TH synthesis (D'Angelo et al., 2016). To test this hypothesis, the hypothyroid animal model was created by treating pregnant mice with PTU. This treatment significantly increased the serum levels of TSH and reduced T4 production in neonates. Melatonin application reversed these alterations induced by PTU. This indicated that melatonin antagonized the adverse effects of PTU on thyroid gland to promote thyroid production. The result was in agreement with the report
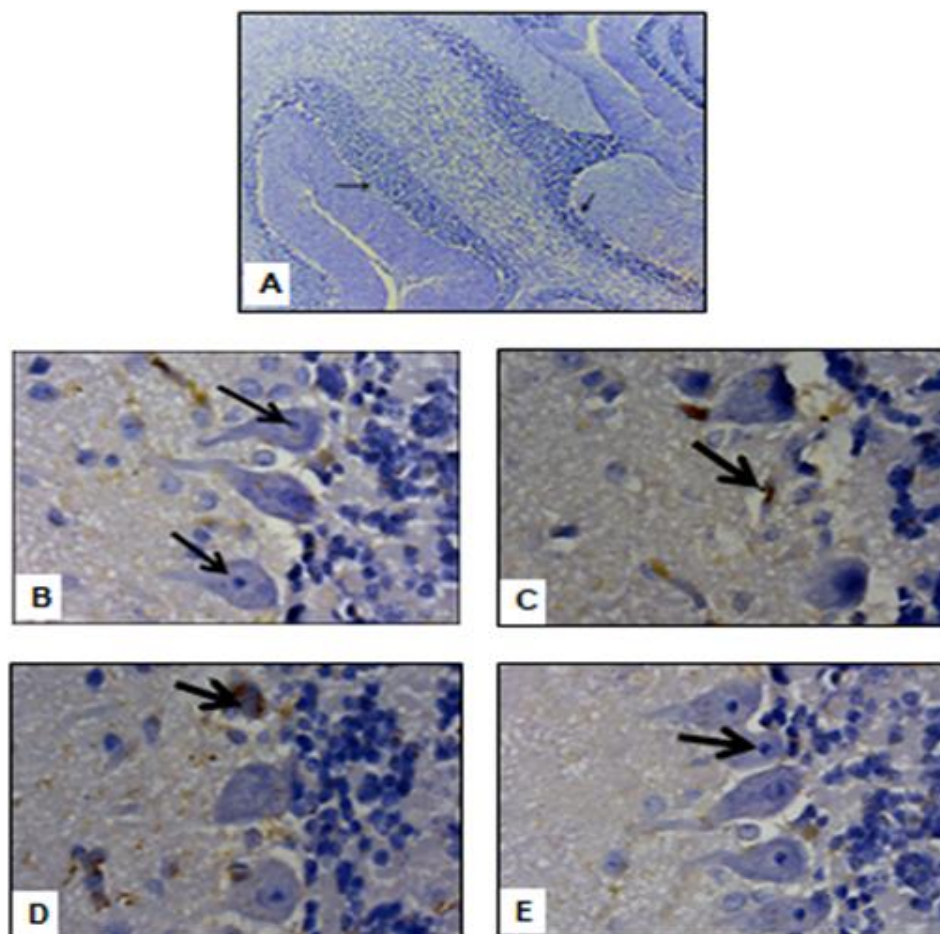
**Figure 4.** Effects of PTU and melatonin on Purkinje cells of cerebellum
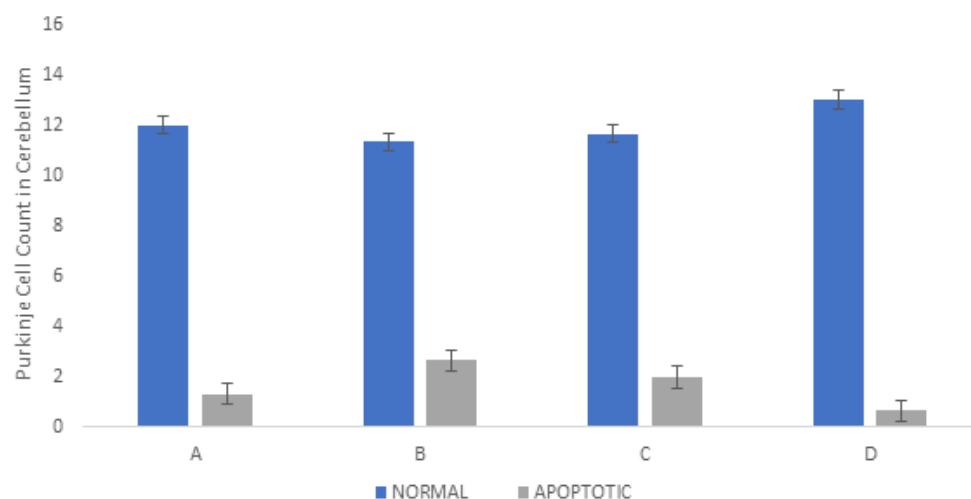
Figure 5. Statistical analysis of the effects of PTU and melatonin on the Purkinje cells in cerebellum of 22 days old pups.

of Garcia-Marin et al. (2011) in which they found that melatonin directly regulated TH biosynthetic activity of cultured rat thyrocytes (Garcia et al., 2011).

THs are conveyed from the mother to the fetus at an early stage of pregnancy before the fetus gains the capacity to synthesize them (Karamitri and Jockers,
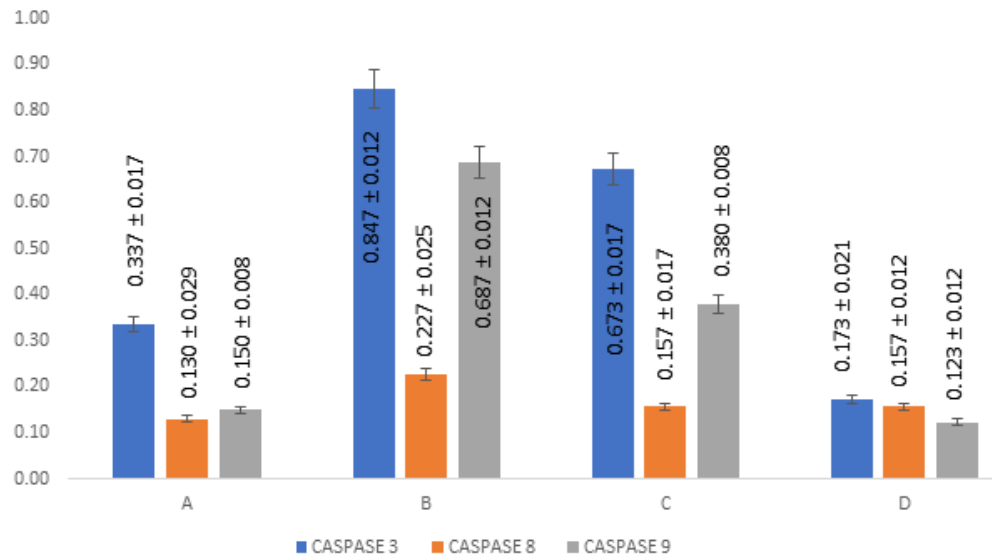
Figure 6. The bar graph of the relative gene expression levels of caspase 3, 8 & 9 under different treatments in 22 days old pups.

2019). The deficiency of THs during brain development leads to morphological alterations and functional disturbance in the motor neurons of cerebral cortex, hippocampus and cerebellum (Rubenstein, 2011). The effects of THs on brain development have been well documented and it has been proved that TH deficiency will result in retarded brain development in neonates (Koibuchi and Chin, 2000; Darras, 2019; Dussault and Ruel, 1987).

In the present study, the effects of the maternal TH deficiency on the neuronal damage of the neonate and the potential protective mechanisms of melatonin on it were tested. It has been known for many years that THs regulate the development of cerebellum (Faustino and Ortiga-Carvalho, 2014). The cerebellar development is mostly postnatal in both humans and rodents and various anatomical modifications induced by TH deficiency in rodents have been well acknowledged and it has been documented that the same morphological changes are observed in the human neonatal brains too (Leto et al., 2016). In the case of Purkinje cells observed in the current study, viable neurons had a lightly stained spherical nucleus (Figure 4B), whereas apoptotic neurons showed shrunken fragmented nuclei, clearly visible in hypothyroid group (Figure 4C). There was not much difference seen in normal cell count in all the 4 groups, but in the case of apoptotic cell count, PTU treated group B had a higher count as compared to control A and melatonin treated groups C and D (Figure 5). Ahmed et al (2014) observed that PTU administration in neonatal rats caused a marked reduction in Purkinje cell count along with histological changes, including cell edema and cellular fragmentation (Ahmad et al., 2014). No work has been done so far to observe the effects of melatonin on the histology of Purkinje cells. This was the

first time to record histological conservation of features of the cell nucleus and the Purkinje cell as a whole by administration of melatonin to hypothyroid pups. The restoration of cell structure and number was more prominent and significant in group C (Figure 4D), indicating that melatonin works well when administered along with PTU.

Compared to humans, rat pups are born with morphologically immature brain (Bhanja and Jena, 2013). It is in the early postnatal period of 21 days that the THs effect on morphology of the brain are observed (Bernal, 2005; Koibuchi and Iwasaki, 2006). This stage of maturation in rats parallels the last trimester of human pregnancy. In both humans and rodents, delay in introducing THs leads to permanent structural and functional impairment of the brain (Bernal, 2005; Koibuchi and Iwasaki, 2006).

Loss of mitochondrial barrier function in the 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 (Wang, 2009). Caspase-9, which forms part of the pathway signaling through the mitochondria, showed an increase in expression in group B as compared to other groups (Figure 6). The initiator caspase 9 then activates the extrinsic caspases, such as caspase 3, which bring about the apoptotic destruction of the cell. Likewise, expressions of caspase 3 were significantly higher as compared to 8 and 9 in group B (Figure 6) when compared to other groups. Melatonin treated C group showed a significant reduction in the expressions of caspase 9 and 3, highlighting the anti-apoptotic effect of this hormone on postnatal day 21 (Figure 6). In contrast, caspase 8, which forms part of the extrinsic apoptotic pathway, showed no remarkable change in expressions

of any of the groups (Figure 6).

Many extensive studies on postnatal development of cerebellum have been done so far, as each type of cell in this part of the brain is sensitive,in one way or the other, to the lack of THs. For example, the inward migration of the granular cell precursors present in the granular layer cells is inhibited, the development of the dendritic arborization of Purkinje neurons is impaired, and the maturation of the GABAergic interneurons is delayed (Morte et al., 2002). The proliferation and differentiation of glial cells, including astrocytes, oligodendrocyte precursors, and microglia are affected (Morte et al., 2002). So far, there have been no reports regarding the effect of PTU in causing apoptotic changes in the Purkinje neurons and the effects of melatonin on these changes during intrauterine life or postnatally.

In the current study, it was observed that maternal hypothyroidism caused shrinkage of the nucleus and positive Bax stain of Purkinje cells of cerebellum. Melatonin treatment significantly reduced hypothyroid associated apoptosis in this area (Figure 4D). This observation is consistent with the previous report regarding the effect of hypothyroidism on the development of Purkinje cells by Ahmed et al. (2014), but no work has been reported regarding the role of melatonin on hypothyroidism induced apoptosis in Purkinje cells.

Conclusion

Since there are limited remedies to effectively treat hypothyroidism related neonatal brain damage, the present results may lead to the development of potential treatment strategies regarding the use of melatonin in the hypothyroid mother.

There is a 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 may help to maintain neuronal function and survival. It is assumed that these findings will have significant applications.

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