Research Article

Protective effects of melatonin on mitochondrial injury and neonatal neuron apoptosis induced by maternal hypothyroidism

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ABSTRACT

   In the current study, we reported the beneficial effects of melatonin in preventing neonatal neuronal apoptosis induced by maternal hypothyroidism. During the gestation and early lactation stages, the mother rats were given propylthiouracil (PTU) to inhibit their thyroid gland activity which resulted in the increased serum TSH and reduced T4 levels. This maternal hypothyroidism caused neuronal apoptosis of their pups, particularly in the CA3 area of hippocampus. Melatonin administration preserved function of thyroid gland and significantly reduced the apoptosis. Further studies have uncovered the potentially protective mechanisms of melatonin, that is, as a mitochondrial targeted antioxidant, melatonin preserves the mitochondrial outer membrane, inhibits the release of cytochrome C from mitochondria to cytoplasm and down regulates the gene expressions of Bax, along with caspases 3 and 9. Thus, melatonin breaks the mitochondria related apoptotic pathway to suppress the neuronal apoptosis induced by the maternal hypothyroidism. Considering the limited remedies to effectively treat hypothyroidism associated neonatal brain damage, melatonin may provide an alternative method for this disorder.

Keywords: Melatonin, mitochondria, propylthiouracyl, hypothyroidism, apoptosis, cytochrome c oxidase, thyroid stimulating hormone, thyroid hormones.

1. INTRODUCTION

   Maternal hypothyroidism impairs development of various tissues and organs of fetus throughout pregnancy (1). The fetal brain is the most liable organ to be impacted by the imbalance of thyroid hormones (THs) during the first trimester in which the maternal thyroid gland is the only source of this hormone (2). TH receptors are found in highest concentration in developing neurons of fetal brain which includes cerebellum and cerebrum, especially the hippocampus (3). Hippocampus and prefrontal cortex are two structures associated with learning and memory and are interrelated to each other directly (4). The hippocampus, by evolution, is

one of the oldest parts of the brain (5). It is located in the temporal lobe and is an integral component of the limbic system (5). It consists of the dentate gyrus and Cornu Ammonis (CA) (5, 6). CA is anatomically and functionally separated into distinct subfields named CA1, CA2, CA3 and CA4 (6). The CA3 region has been the major focus of researchers in recent years for its specific role in memory processes. Neuronal connection in the CA3 area is much denser than that of other areas (7, 8). Axon collaterals of CA3 pyramidal cells make extensively excitatory connections with adjacent neurons (8). Thus, hippocampus is vulnerable to a variety of stimuli and susceptible to neuro-degeneration (7, 8). It has been known that hypothyroidism has adverse effects on cerebrum (9), cerebellum (10, 11) and particularly on hippocampus (12, 13). Importantly, during stage of neuronal development, reduction or absence of TH leads to alterations in molecular organization, morphology and functions in the pyramidal and purkinje neurons associated with apoptosis (14). Apoptosis is an indispensable physiological process for the development of the nervous system, but concomitantly, excessive apoptosis, for example, in neurologic injury or some other neuronal disorders, also leads to pathological loss of neurons (15). Neurons are instructed to live or die by certain extracellular and intracellular stimuli. These include exposure to neurotoxins, oxidative stress, and DNA damage (16). These stimuli prompt the signaling pathways that are assimilated at the mitochondrial apoptotic machinery, resulting in cell survival or death. When neurons are exposed to excessive oxidative stress, they may respond by either overcoming the stress or end up with apoptosis (17).

Mitochondria are the most important organelles and their status can determine the outcomes of the cell fates. Not only do they provide the biological energy for carrying out the essential activities of the cell but also initiate the death signal for apoptosis (18, 19). It has been reported that TH deficiency during neurogenesis causes alteration in mitochondrial morphology, which eventually causes mtDNA damage, leading to extensive apoptosis and reduced sizes and numbers of neurons (20). Much research work has been done highlighting the reactive oxygen species (ROS) as a significant factor in mitochondrial dysfunction linked with apoptosis. The deficiency of maternal TH during gestation has also been attributed as a major cause of neuronal deficit in infants born to the hypothyroid mothers (21, 22).

As to the apoptosis, caspases are the principal effectors to initiate this process. They are referred as the killer molecules. Upon their activation these caspases will degrade cytoplasmic and nuclear processes, thereby, promoting apoptotic cell death (23). They reside in the cytoplasm of cells as the inactive zymogens. Breakdown of these zymogens generates active enzymes that trigger apoptosis. Many experiments have confirmed the significant role of caspases in loss of neurons after induction of ischemia (24, 25). One of the major pathways for caspase activation involves the participation of mitochondria. In this pathway, mitochondria release cytochrome C (cyto C) into cytoplasm. Discharge of cyto C from the intermembrane space of mitochondria occurs usually on the contact of neurons to the apoptotic stimuli (26). To suppress the cyto C discharge from the mitochondria is an effective approach to reduce the neuronal apoptosis (27-29). Melatonin is one of the molecules to have this capacity.

Melatonin, synthesized in the pineal gland and other organs, is categorized as an effective antioxidant and anti-apoptotic molecule that specifically targets mitochondria (30). Recently, it has been reported that melatonin is mainly synthesized in mitochondria (31) and the mitochondrial matrix is the exclusive site for melatonin synthesis in neurons of mice (32). These characteristics of melatonin render it effectively reducing mtDNA damage (33). Numerous studies have documented melatonin's anti-apoptotic effects (34, 35) by improving mitochondrial membrane and cell membrane stability (36). Interestingly, the brain tissue has much higher

melatonin level than that of other tissues in the body (37). It is even more important that the highest melatonin level has been identified in the mitochondria among other cellular compartments (38). A wide range of *in vivo* and *in vitro* studies have confirmed that melatonin targets mitochondria to reduce their oxidative stress and thus, results in decreased apoptosis and improved metabolic status of cells (39, 40). Since binding sites for melatonin have been found in the hippocampus of several mammals, it has been suggested that this molecule is able to modulate neuronal functions of hippocampal cells (41).

In mitochondria, cyto C is a typical constituent of the electron transport chain. It is tethered to inner mitochondrial membrane. Under the oxidative stress, cyto C leaks into the cytoplasm (42) and initiate the process of apoptosis. The mechanism is that leaked cyto C binds to converter protein Apaf-1 and pro-caspase-9 (43) in the cytoplasm. Consequently, this protein complex initiates executioner caspases such as caspase-3, leading to apoptosis.

Considering the critical role of mitochondria played in cellular energetic metabolism and apoptosis (44, 45) and the protective effects of melatonin on mitochondria, the current study investigated the outcome of melatonin on the mitochondrial injury in the neonatal brains which are exposed to maternal hypothyroidism. Focus was given to the hippocampus and moreover, the gene expressions of caspase 3, 8 and 9 was measured to identify the potential mechanisms of apoptosis induced by the maternal hypothyroidism.

2. MATERIALS AND METHODS

2.1. Animals.

The animals were purchased from University of Veterinary Animal Sciences, Lahore, Pakistan. The animal study was performed in the Experimental Research Lab and Department of Anatomy of University of Health Sciences (UHS), Lahore, Pakistan. 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 approved protocol number for this study is UHS/ERC/126-17/19. The animals were housed in the temperature-controlled room with $22 \pm 1^{o}C$ 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 acclimation for these animals.

2.2. Study procedures.

Twelve apparently healthy female Wistar rats, 12-16 weeks, in the weight range of 200-250 g, were divided equally into four groups. Group A served as control and received plain drinking water and chow. Group B received 15mg/kg/day of propylthiouracyl (PTU) (46) orally mixed with chow. Group C received PTU plus melatonin (10mg of melatonin/kg/day in drinking water (47). 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 to get pregnancy. The treatment continued throughout the period of gestation and weaning. During the period of experiment, the serum levels of TSH, T3 and T4 of the dams were measured weekly. After delivery, the pups were allowed free access to maternal milk. A total of 40 pups, 10 from each group, were used in the study. All the pups were sacrificed on 22nd day of their life.

2.3. Analysis of Serum TSH, T3 and T4.

Blood samples were collected from the cardiac region for immediately evaluating serum levels of TSH, T3 and T4 using Elisa kit following the instructions of the manufacturer. The parameters related to mitochondria were carried out only after confirming the values of serum TSH, T3 and T4 in all experimental groups.

2.4. Immunohistochemical analysis of apoptosis in CA3 area of Hippocampus.

The 3 μm thick sections of hippocampus mounted on slides were incubated in oven at 60ºC for an hour and then, processed with 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 water bath at 60 ºC 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 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 (3,32 -Diaminobenzidine) 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 pyramidal neurons from CA3 region of hippocampus were analyzed under light microscope. Briefly, a total of 3 sections from each slide (15 sections/ animal) were photographed in each CA3 area of the hippocampus.

The pyramidal cell count was carried out by over the ocular graticule on the CA3 area with a calibration factor of 40 µm. The neurons inside the squares of the grid were recorded except the cells lying on 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 size of the cell body, fragmentation of the nucleus, extent of Bax staining of the cell nucleus or cytoplasm. Pyramidal cell count was expressed as number of pyramidal cells per mm^2 for both normal and apoptotic cells.

2.5. Preparation of mitochondria from brain tissue.

Neonatal brain was extracted from the skull. A portion of 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 EGTA and 5 mM Hepes at pH 7.4. The homogenized tissue was used for isolation for both RNAs and mitochondria with the help of RNAs isolation kit (FavorPrep Tissue Total RNA Mini Kit, Catalog #FATRK001, Taiwan) and mitochondrial isolation kit (Abnova kit, Catalog #KA0895), respectively. Out of the total of 0.3 – 0.4 g of brain tissue extracted, one half of the homogenate was centrifuged at 1,000xg for 10 minutes at 4ºC. The supernatant was collected and further centrifuged at 12,000xg for 15 min at 4 ºC.   Then, 0.5 ml of isolation buffer from the mitochondrial isolation kit and 5µl of protease inhibitor cocktail were added to each pellet and

the mixture was centrifuged at 12,000xg for 15 min at 4 ⁰C to obtain mitochondria. The freshly isolated mitochondria were used for further experiment.

2.6. Isolation and extraction of RNA.

The whole process of RNA extraction was performed under cold environment and sterile conditions. Extraction and isolation of RNA was 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. One half of the homogenate was centrifuged at 1,000xg for 10 min at 4°C. The pellet was discarded and the supernatant was collected and further centrifuged at 12,000xg for 15 min at 4 °C. 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 4 ⁰C, the precipitants were frozen at -80 ⁰C until use. The primers were designed using Gene Bank sequences for caspase 3, 8, and 9 (Table 1).

2.7. 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 run by the ΔΔCt method.

2.8. Measurement of cyto C oxidase activity outside of mitochondria.

The quantitative spectrophotometric method was used to detect the absolute concentration of cyto C outside of mitochondria. A decrease in absorbance of ferrocytochrome C at 550 nm is caused by its oxidation to ferricytochrome C by cyto c oxidase. Enzymatic activity of oxidized cyto C was determined spectrophotometrically with the help of cyto c oxidase assay kit (TriBioScience Catalog Number TBS2115, Sunnyvale, CA, USA) at 550 nm at 25 °C in 96-well plates (48, 49). A detergent, n-Dodecyl β-d-maltoside, was added in solution to rupture mitochondrial membrane to measure the total activity of cyto c oxidase in the mitochondrial suspension. The reliability of the outer membrane of mitochondria was calculated by determining the ratio between cyto c oxidase activity of mitochondria in the presence or absence of the detergent, n-dodecyl β-d-maltoside (49).

2.9. Spectroscopic determination of cyto C oxidase in the intact mitochondria.

Cyto C oxidase activity in the mitochondrial pellet was also measured as a marker of mitochondrial activity and the integrity of mitochondrial outer membrane. For each sample, 1 to

2 µg mitochondria suspension was mixed with either assay buffer (measuring cyto c oxidase activity in intact mitochondria) or with the assay buffer containing nDodecyl β-D maltoside (measuring total Cyto C oxidase activity). Activity was calculated as [optical density (ΔOD)/time (Δt, min)] (50). Percentage of mitochondria with intact outer membrane was calculated in all the groups by applying the formula:

$$\frac{A/minute \text{ (with detergent)} - A/minute \text{ (without detergent)} \times 100}{A/minute \text{ (with detergent)}}$$

(where A= absorbance reading at 550nm by spectrophotometer)

The readings were recorded at 550 nm (51). Data were analyzed and stored with the use of a data management system. The maximum linear rate for each sample was calculated to assess the degree of mitochondrial integrity.

2.9. Statistical analyses.

Data was presented as mean ± SD. One way analysis of variance (ANOVA) was used for normality analysis among groups, followed by Tukey's post-hoc analysis to obtain the statistical significance of differences 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.

3. RESULTS

3.1. The sequences of the primers of caspases 3, 8 and 9.

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

3.2. Effects of PTU and melatonin on the serum levels of TSH, T3 and T4.

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 TSH levels are comparable to 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 groups (Figure 2). In contrast, serum total T4 level was significantly lower in PTU treated animals than that in the control animals. Melatonin alone did not alter the T4 level. However, in the animals co-treated with PTU and melatonin, their T4 level was significantly elevated compared to the only PTU treated animals (p < 0.05) (Figure 2).
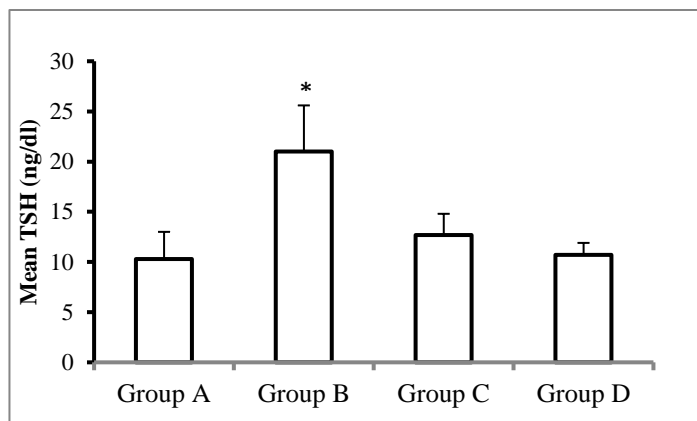
Fig. 1. Effects of PTU and melatonin on the serum levels of TSH in neonates.

*The data were expressed as mean ± SD (n = 10). Group A: control, Group B: treated with PTU, Group C: treated with PTU plus melatonin, Group D: melatonin treated alone. * p < 0.05 vs other groups.*
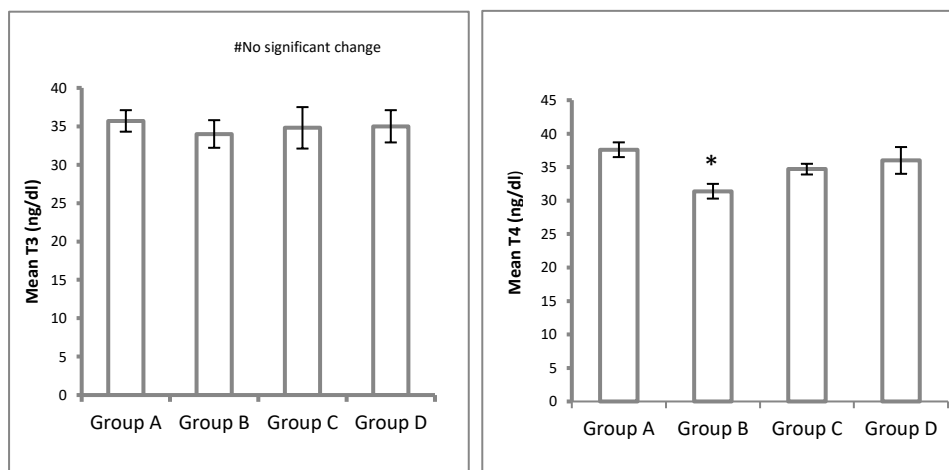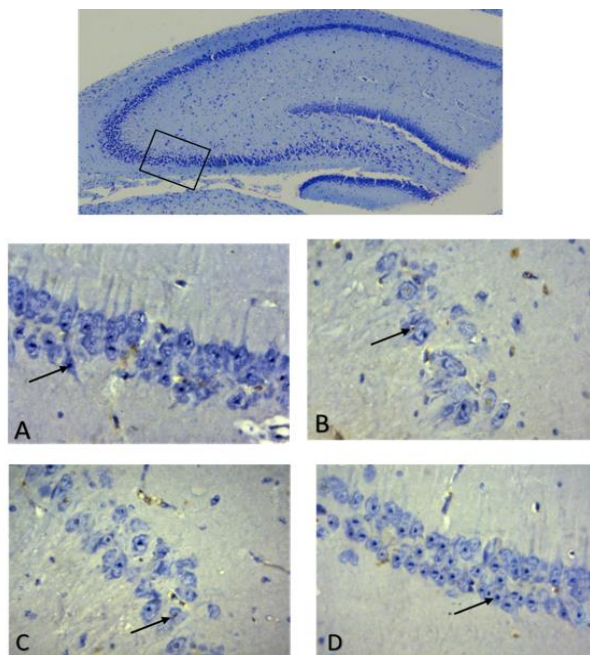

Fig. 2. Effect of PTU and melatonin on the serum levels of T3 and T4 in neonates.

*The left panel: T3, right panel T4. The data were expressed as mean ± SD (n = 10). Group A: control, Group B: treated with PTU, Group C: treated with PTU plus melatonin, Group D: melatonin treated alone. * p < 0.05 vs other groups.*
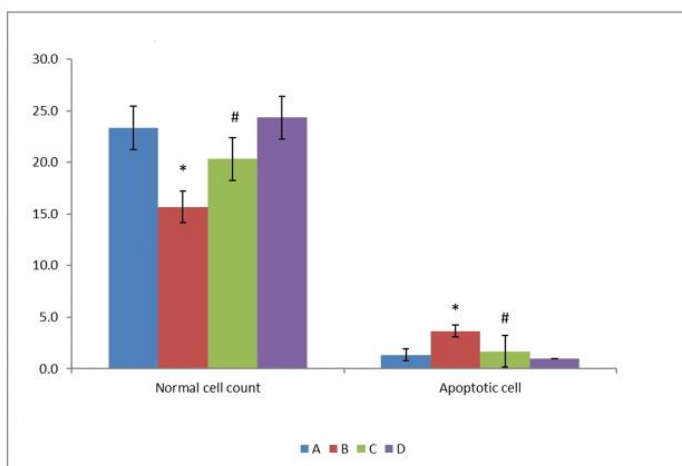
3.3. Effects of PTU and melatonin on the apoptosis of pyramidal cells in CA3 area of hippocampus.

The expression of Bax, an index of apoptosis, in the nucleus and cytoplasm of pyramidal cells in CA3 area of hippocampus was significantly upregulated in PTU group compared to control group (Figure 3). The nucleus was shrunken and pyknotic (Figure 3B and 4). Melatonin treatment significantly reduced the neuronal apoptosis induced by the PTU (Figure 3C and 4). Melatonin alone preserved the neurons as they were in the control group (Figure 3D and 4). The statistical analyses on the normal neurons and apoptotic neurons in this area were illustrated in Figure 4.

Fig. 3. Effects of PTU and melatonin on the apoptosis of pyramidal cells in CA3 area of hippocampus.

Immunohistochemically staining of Bax (40x magnification) from CA3 area of hippocampus. The square in top most panel indicated the CA3 area and the panel A, B, C and D were the magnified CA3 areas of each treatment. A: control, B: PTU, C: PTU plus melatonin, D: melatonin alone. Arrows in B and C pointed the pyknotic nuclei with Bax staining. The structures of cells are also disturbed, however, the damage in B was significantly intense compared to the C. The arrows in A and D pointed to the normal nuclei.

Fig. 4. Statistical analysis of the effects of PTU and melatonin on the apoptosis of pyramidal cells in CA3 area of hippocampus.

*The data were expressed as mean ± SD (n = 10). A: control, B: treated with PTU, C: treated with PTU plus melatonin, D: melatonin treated alone. * p < 0.05 VS A, C and D groups, # < 0.05 vs A, B and D groups.*
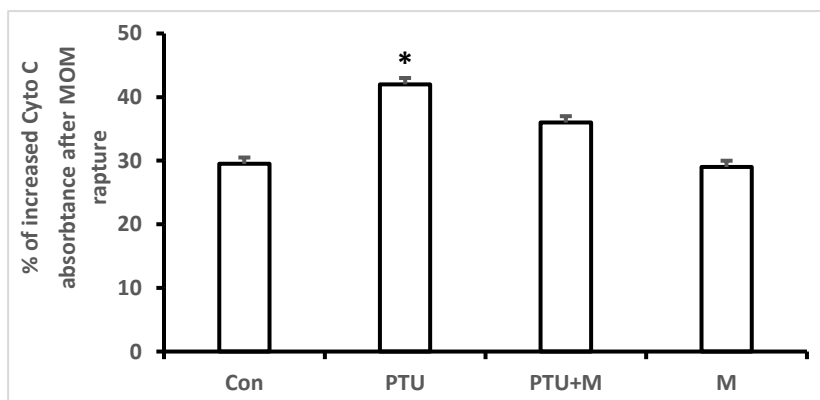
3. 4. Effects of PTU and melatonin on the mitochondrial outer membrane.

In this experiment, detergent nDodecyl β-D maltoside was used to rupture the mitochondrial outer membrane to expose cyto C oxidase for its activity measurement since membrane rupturing would result in cyto C oxidase to be translocated from mitochondria into the cytosol. The results were listed in Table 2. The results showed that under the condition of assumed intact of mitochondrial outer membrane, the highest activity of cyto C was found in the PTU treated animals. The higher the value indicated, the more the injury of the mitochondrial outer membrane. After the mitochondria were treated with nDodecyl β-D maltoside to rupture outer membrane of mitochondria, the cyto C absorbances were significantly increased in all groups. However, the highest increase was still observed in the PTU group in which, the cyto C absorbance increased 41%. The rest groups are 36, 29 and 28% for PTU plus melatonin, control and melatonin alone groups, respectively (Table 2 and Figure 5).

Table 2 - **Cyto C oxidase activity indicated by cyto C absorbance at 550 nm among groups.**

Group	cyto C absorbance ratio at 550nm		Increased % before and after outer membrane rupture
	Intact mitochondrial outer membrane	Ruptured mitochondrial outer membrane	
A	0.46 ± 0.056	0.60 ± 0.049	29.5 ± 0.92
B	0.69 ± 0.002	0.98 ± 0.010	$41.8 \pm 0.68*$
C	0.59 ± 0.001	0.82 ± 0.021	36.2 ± 0.33
D	0.41 ± 0.002	0.52 ± 0.001	28.8 ± 0.05

*Data is expressed as mean $\pm$ S.D (n = 10) * P < 0.001 indicated highly significant difference vs other groups. Group A: control, Group B: treated with PTU, Group C: treated with PTU plus melatonin, Group D: melatonin treated alone.*

Fig. 5. Effect of PTU and melatonin on the rupture of mitochondrial outer membrane induced by nDodecyl β-D maltoside.
*The data were expressed as mean $\pm$ SD (n =10). MOM: mitochondrial outer membrane, Con: control, PTU: propylthiouracyl treated group PTU+M: propylthiouracyl plus melatonin, M: melatonin treated alone. * indicated statistical significance vs the relative group.*

3.4. Effects of PTU and melatonin on the caspases 3, 8 and 9.

The levels of mRNA expressions of the caspases 3 (32kD), caspase-8 (55kD) and caspase 9 (45kD) after normalized with a house keeping gene, β-Actin, mRNA, are shown in Table 3 and Figure 6. The statistical analyses showed that the PTU treatment significantly increased gene expressions of caspase 3 and especially, the caspase 9. No significant effect on caspase 8 had been observed among groups. However, if the animals were co-treated with PTU plus melatonin, melatonin effectively suppressed gene expressions of caspases 3 and 9 upregulated by the PTU. Melatonin treatment alone had no significant influence on gene expressions of all the caspases tested (Table 3 and Figure 6)

Table 3. The gene expression levels of caspase 3, 8, 9 under different treatments.

Groups	caspase 3	caspase 8	caspase 9
A	0.33	0.13	0
B	0.72*	0.23	1*
C	0.50	0.13	0
D	0.29	0.09	0

*The data were expressed as mean ± SD (n=10). The numbers in the table were the altered folds of the gene expression after normalizing the values with a house keeping gene β-Actin mRNA. Group A: control, Group B: treated with PTU, Group C: treated with PTU plus melatonin, Group D: melatonin treated alone. *indicated statistical significance vs the rest of the groups.*
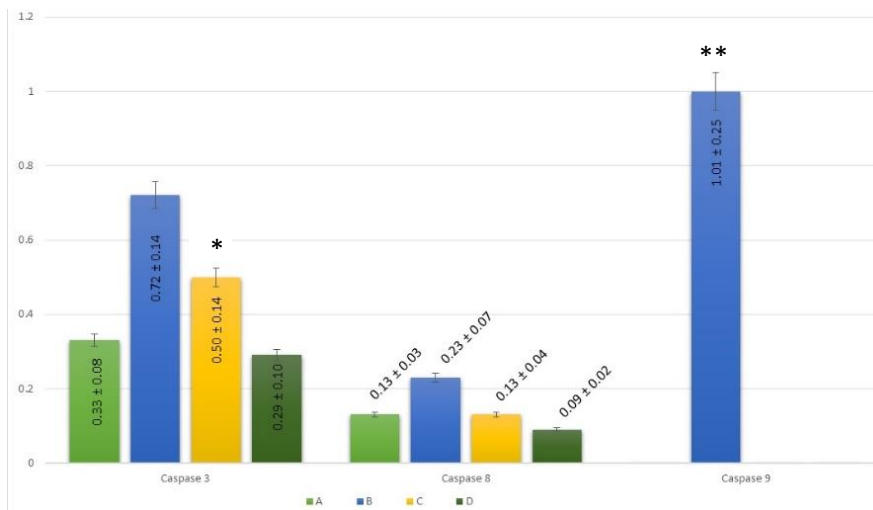
Fig. 6. The bar graph of the relative gene expression levels of caspase 3, 8 & 9 under different treatments.
The data were expressed as mean ± SD (n=10). The numbers in the Y axis were the altered folds of the gene expression. Group A: control, Group B: treated with PTU, Group C: treated with PTU plus melatonin, Group D: melatonin treated alone. * $p < 0.05$ vs other groups in the category of caspase 3, ** $p < 0.01$ vs other groups in the category of caspase 9.

4. DISCUSSION

As the important organelles, mitochondria play a central role in the regulation of apoptotic cell death. It has been documented that the disruption of mitochondrial membrane is probably the earliest event leading to the initiation of cellular apoptosis (52). This was confirmed in the current study in which it was observed that THs deficiency during gestation and lactation led to mitochondrial outer membrane injury of neurons from neonates. THs are important regulators to maintain the coordination between mitochondria and nuclei to avoid the unnecessary apoptosis (53, 54). Even a slight shift in the serum levels of THs would lead to disturbed mitochondrial functions and apoptosis (55).

A variety of factors can trigger mitochondria associated apoptosis. These factors include DNA damage, oxidative stress, ultraviolet irradiation, protein kinase inhibition and growth factor deprivation (56). In the present study, initiation of apoptosis in neonatal brain due to maternal TH deficiency was observed. It was known that maternal TH regulates early development of fetal brain in human and animal models (57, 58). Thyroid receptors (TRs) have already been present in the fetal brain even before the commencement of fetal thyroid function, and these receptors are widely spread in the brain to ensure their physiological effects (59, 60).

THs are conveyed from the mother to the fetus at early stage of pregnancy before the fetus gains the capacity to synthesize THs (61). The deficiency of TH during brain development leads to morphological alterations and functional disturbance in the motor neurons of cerebral cortex, hippocampus and cerebellum (62).

The effects of THs on brain development have been well documented (63-66) and TH deficiency will result in retarded brain development in neonates. Since melatonin is a versatile neuroprotector, we hypothesize that melatonin may provide protective effects on hypothyroid related brain damage in neonates, especially, that melatonin has been reported to regulate TH synthesis (67). To test this hypothesis, the hypothyroid animal model was created by treating the pregnant mice with PTU. This treatment significantly increased the serum levels of TSH and reduced T4 production. Melatonin application reversed these alterations induced by PTU. This indicated that melatonin antagonized the adverse effects of PTU on thyroid gland to promote the thyroid production. The result was in agreement with the report of Garcia-Marin *et al.* (68) in which they found that melatonin directly regulated TH biosynthetic activity of cultured rat thyrocytes. Then, we tested the effects of the maternal TH deficiency on the neuronal damage of the neonate and the potential protective mechanisms of melatonin on it. The focus was given to the mitochondria associated neuronal apoptosis. It has been reported that melatonin is a mitochondria targeted antioxidant (69) and it is mainly synthesized and metabolized by mitochondria (70). Melatonin also has a strong association with hippocampus. Mushoff *et al.* (71) reported the effects of melatonin on the spontaneous firing rate of action potentials in the CA1 region of hippocampus. Both the MT1 and MT2 melatonin receptors are also present in the dentate gyrus, CA3 and CA1 regions (72-74). In the current study, we observed that maternal hypothyroidism caused massive cellular destruction and apoptosis in the CA3 area of hippocampus. Melatonin treatment significantly reduced this hypothyroid associated apoptosis in this area. This observation is consistent with the previous report regarding the protective effects of melatonin on the neuronal damage in CA3 area induced by the excitatory toxic kainic acid (75). The mechanistic investigation has found that the apoptosis is related to cyto C. Cyto C is a critical part of the mitochondrial electron transport chain (ETC). It plays a vital role in initiation and regulation of mitochondria associated apoptosis. Cyto C, as an initiator of caspases, triggers

series of events leading to cell apoptosis (76). The caspases activated by cyto C attack mitochondria to release more cytochrome C, a vicious cycle, and eventually, this vicious cycle causes mitochondrial dysfunction with ETC disruption and increase in intracellular reactive oxygen species（ROS） (77). Mansouri *et al.* (78) reported that melatonin diminished the ethanol-induced hepatic mitochondrial DNA damage in mice and this protective effect was attributed to melatonin's antioxidant capability. In 2011, Jou (79) witnessed that melatonin lessened mitochondrial complexes I and IV inhibition induced by ruthenium red and decreased mitochondrial oxidative stress. Melatonin has widespread protective effects on mitochondria. These include reducing mitochondrial oxidative stress (79), preserving mitochondrial membrane (80), upregulation of antiapoptotic mitochondrial protein Bcl2, downregulation of proapoptotic mitochondrial protein Bax (81), increased efficiency of ATP production, decreased release of cyto C into the cytosol and inhibition of caspase 3 activity (82). In the current study, we observed that melatonin downregulates the Bax expression, preserves the integrity of mitochondrial outer membrane and thus, inhibits the release of cytochrome C into the cytosol. In other words, melatonin breaks the vicious cycle of cyto C release-mitochondrial damage and effectively reduces the mitochondria associated apoptosis.

Many studies have documented the adverse effects of hypothyroidism on antioxidant defense system in several regions of the brain (83). It is proposed that the elevation of nitric oxide level induced by hypothyroid state causes oxidative stress leading to neuronal damage (84). Excessive nitric oxide by reacting with superoxide anion forms highly reactive metabolite, peroxynitrite (85). Therefore, this powerful oxidant results in oxidative stress and damages many biological molecules (86). Moreover, excessive nitric oxide causes dysfunction of mitochondria due to the damaging of the complexes of the respiratory chain, including cyto C oxidase, which finally leads to more superoxide anion formation, further accelerating oxidative damage (87, 88).

The mitochondrial outer membrane injury in hypothyroid animals might relate to the activation of the intrinsic pathway of apoptosis. In this pathway, the apoptosis is mediated by mitochondria in which the penetrability of the mitochondrial inner membrane is increased. This phenomenon is known as mitochondrial permeability transition with the opening of pores in the mitochondrial inner membrane (89). Both calcium ions and ROS are major regulators of mitochondrial permeability (90, 91). Disproportion of both of these factors eventually leads to mitochondrial swelling, loss of respiratory control, and a release of matrix calcium into the cytosol and later, apoptosis. Our results showed that the hypothyroid state did not modify the gene expression of caspase 8, which is the indicator of extrinsic pathway of apoptosis. This suggests that the hypothyroid induced neuronal apoptosis is not associated with the extrinsic pathway of apoptosis. However, the gene expressions of caspases 3 and 9 were upregulated. Both of them are involved in the mitochondrial-mediated apoptosis. This was also further confirmed that the melatonin's protective effects mainly targeted on mitochondria. Our findings confirmed that melatonin preserved mitochondrial membrane potential by inhibition of mitochondrial permeability transition pore and therefore stabilized its membrane and inhibited apoptosis (92).

In conclusion, our results showed that maternal hypothyroidism caused neuronal apoptosis of the neonates via the mitochondrial pathway. Melatonin by its antioxidant capacity stabilized the mitochondrial outer membrane and inhibited release of cyto C into the cytoplasm, thus, it suppressed the initiation of mitochondria associated apoptosis in TH deficient neonatal brain. This was indicated by the reduction in the structural destruction and apoptosis in the Pyramidal neurons of CA3 area in Hippocampus. Since there are limited remedies to effectively treat

hypothyroidism related neonatal brain damage our results may lead to the development of potential treatment strategies related to the use of melatonin in the hypothyroid mother.

ABBREVIATIONS

cyto C – Cytochrome C oxidase
TH – Thyroid Hormone
TSH – Thyroid Stimulating Hormone
UHS – University of Health Sciences
mtDNA – Mitochondrial DNA
EGTA (Ethylene Glycol Tetraacetic Acid)
ROS – Reactive Oxygen Species
HRP – Horse Reddish Peroxidase
DPX - Dinbutylphthalate Xylene
RT-qPCR – Real Time Quantitative Polymerase Chain Reaction

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AUTHORSHIP

Mariyah Hidayat conceived, designed and did statistical analysis and editing of manuscript. Saba Khaliq and Adullah Khurram did data collection and manuscript writing. Prof. Dr Khalid Pervaiz Lone designed, reviewed and approved the manuscript

CONFLICT INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N & Buss C (2015) Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience* **342**: 68-100.
2. Bernal J (2015) Thyroid hormones in brain development and function. In: Feingold KR *et al*. (ED). *Endotext*. https://www.ncbi.nlm.nih.gov/books/NBK285549.
3. Escobar GM, Obregon MJ, Escobar del Re F (2007) Iodine deficiency and brain development in the first half of pregnancy. *Public Health Nutrition* **10** (12A): 1554–1570 doi: 10.1017/S1368980007360928.
4. Jin J, Maren S (2015) Prefrontal-hippocampal interactions in memory and emotion. *Front. Syst. Neurosci.* **9**: 170. doi:10.3389/fnsys.2015.00170.
5. Cherubini E, Miles R (2015) The CA3 region of the hippocampus: how is it? What is it for? How does it do it? *Front. Cell Neurosci*. **9**: 19. doi:10.3389/fncel.2015.00019.

6. Amaral DG, Scharfman HE, Lavenex P (2007) The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). *Prog. Brain Res.* **163**: 3–22. doi:10.1016/S0079-6123(07)63001-5.

7. Maruszak A, Thuret S (2014) Why looking at the whole hippocampus is not enough—a critical role for anteroposterior axis, subfield and activation analyses to enhance predictive value of hippocampal changes for Alzheimer's disease diagnosis. *Front. Cell Neurosci.* **8**: 95. https://doi.org/10.3389/fncel.2014.00095.

8. Le Duigou C, Simonnet J, Teleñczuk MT, Fricker D, Miles R (2014) Recurrent synapses and circuits in the CA3 region of the hippocampus: an associative network. *Front. Cell Neurosci.* **7**: 262. doi:10.3389/fncel.2013.00262.

9. Hidayat M, Haider I, Khurram A, Lone KP (2019) The immunohistochemical localization of Bax in the brain of hypothyroid neonate during maternal melatonin intake. *Int. J. Anat. Res.* **7** (3): 6901-6905. doi: https://dx.doi.org/10.16965/ijar.2019.259.

10. Xiao Q, Nikodem VM (1998) Apoptosis in the developing cerebellum of the thyroid hormone deficient rat. *Front. Bio-science* **3** (1): A52-57.

11. Faustino LC, Ortiga-Carvalho TM (2014) Thyroid hormone role on cerebellar development and maintenance: a perspective based on transgenic mouse models. *Front. Endocrinol. (Lausanne).* **5**: 75. doi:10.3389/fendo.2014.00075.

12. Cooke GE, Mullally S, Correia N, O'Mara SM, Gibney J (2014) Hippocampal volume is decreased in adults with hypothyroidism. *Thyroid* **24** (3): 433-40. doi: 10.1089/thy.2013.0058.

13. Nam SM, Kim JW, Yoo DY *et al*. (2018) Hypothyroidism increases cyclooxygenase-2 levels and pro-inflammatory response and decreases cell proliferation and neuroblast differentiation in the hippocampus. *Mol. Med. Rep.* **17** (4): 5782–5788. doi:10.3892/mmr.2018.8605.

14. Becker EB, Bonni A (2004) Cell cycle regulation of neuronal apoptosis in development and disease. *Prog. Neurobiol.* **72** (1):1-25.

15. Chatonnet F, Picou F, Fauquier T, Flamant F (2011) Thyroid hormone action in cerebellum and cerebral cortex development. *J. Thyroid Res.* **2011**: 145762. doi:10.4061/2011/145762.

16. Osellame LD, Blacker TS, Duchen MR (2012) Cellular and molecular mechanisms of mitochondrial function. *Best Pract. Res. Clin. Endocrinol. Metab.* **26** (6): 711–723. doi:10.1016/j.beem.2012.05.003.

17. Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35** (4): 495–516. doi:10.1080/01926230701320337.

18. Chen X, Guo C, Kong J (2012) Oxidative stress in neurodegenerative diseases. *Neural Regen. Res.* **7** (5): 376–385. doi:10.3969/j.issn.1673-5374.2012.05.009.

19. Alemu A, Terefe B, Abebe M, Biadgo B (2016) Thyroid hormone dysfunction during pregnancy: A review. *Int. J. Reprod. Biomed. (Yazd).* **14** (11): 677–686.

20. Miranda A, Sousa N (2018) Maternal hormonal milieu influence on fetal brain development. *Brain Behav.* **8** (2): e00920. https://doi.org/10.1002/brb3.920.

21. Sahay RK, Nagesh VS (2012) Hypothyroidism in pregnancy. *Ind. J. Endocrinol. Metab.* **16** (3): 364–370. doi:10.4103/2230-8210.95667.

22. Dirar AM, Kalhan A (2018) Hypothyroidism during pregnancy: Controversy over screening and intervention, *World J. Obstet. Gynecol.* **7** (1): 1-16. doi: 10.5317/wjog.v7.i1.

23. Krajewski S, Krajewska M, Ellerby LM *et al*. (1999) Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proc. Natl. Acad. Sci.* **96** (10): 5752-5757. https://doi.org/10.1073/pnas.96.10.5752.

24. Srinivasan V, Spence DW, Pandi-Perumal SR, Brown GM, Cardinali DP (2011) Melatonin in mitochondrial dysfunction and related disorders. *Int. J. Alzheimers Dis.* **2011**: 326320. doi: 10.4061/2011/326320.

25. Wang C, Youle RJ (2009) The role of mitochondria in apoptosis. *Ann. Rev. Genet.* **43**: 95–118. doi:10.1146/annurev-genet-102108-134850.

26. Rebecca A. Kirkland and James L (2001) Evidence for redox regulation of cytochrome c release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition. *J. Neurosc.* **21** (6): 1949-1963; doi: https://doi.org/10.1523/JNEUROSCI.21-06-01949.2001.

27. Skulachev VP, Anisimov VN, Antonenko YN (2009) An attempt to prevent senescence: A mitochondrial approach. *BBA.* **1787** (5): 437-461 https://doi.org/10.1016/j.bbabio.2008.12.008.

28. Jiang X, Wang X (2004) Cytochrome C-mediated apoptosis. *Ann. Rev. Biochem.* **73**: 87-106.

29. Tan DX, Reiter RJ (2019) Mitochondria: the birth place, battle ground and the site of melatonin metabolism in cells. *Melatonin Res.* **2** (1): 44-66. doi: https://doi.org/10.32794/mr11250011.

30. Beriwal N, Namgyal T, Asmaa PS, Al Quraan M (2019) Role of immune-pineal axis in neurodegenerative diseases, unraveling novel hybrid dark hormone therapies. *Heliyon.* **5** (1): e01190. doi: 10.1016/j.heliyon.2019.e01190.

31. Reiter RJ, Tan DX, Rosales-Corral S, Galano A, Jou MJ, Acuna-Castroviejo D (2018) Melatonin mitigates mitochondrial meltdown: interactions with SIRT3. *Int. J. Mol. Sci.* **19** (8): 2439. doi:10.3390/ijms19082439.

32. Di Bella G, Mascia F, Gualano L, Di Bella L (2013) Melatonin anticancer effects: review. *Int J. Mol. Sci.* **14** (2): 2410–2430. doi:10.3390/ijms14022410.

33. Bojková B, Kubatka P, Qaradakhi T, Zulli A and Kajo K (2018) Melatonin may increase anticancer potential of pleiotropic drugs, *Int. J. Mol. Sci.* **19**: 3910. doi:10.3390/ijms19123910.

34. Tan DX, Manchester LC, Qin L, Reiter RJ (2016) Melatonin: a mitochondrial targeting molecule involving mitochondrial protection and dynamics. *Int. J. Mol. Sci.* **17** (12): 2124. doi:10.3390/ijms17122124.

35. García G, Martínez-Ballarín G, Acuña-Castroviejo D *et al*. (2011). Melatonin reduces membrane rigidity and oxidative damage in the brain of SAMP(8) mice. *Neurobiol. Aging* **32**: 2045-2054. 10.1016/j.neurobiolaging.2009.12.013.

36. Tan DX, Manchester LC, Sanchez-Barcelo E, Mediavilla MD, Reiter RJ (2010) Significance of high levels of endogenous melatonin in mammalian cerebrospinal fluid and in the central nervous system. *Curr. Neuropharmacol.* **8** (3): 162–167. doi:10.2174/157015910792246182.

37. Wang DD, Jin MF, Zhao DJ, Ni H (2019) Reduction of mitophagy-related oxidative stress and preservation of mitochondria function using melatonin therapy in an HT22 hippocampal neuronal cell model of glutamate-induced excitotoxicity. *Front. Endocrinol. (Lausanne).***10**: 550. doi:10.3389/fendo.2019.

38. Webster KA (2012) Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. *Future Cardiol.* **8** (6): 863-884.

39. Schenkel LC, Bakovic M (2014) Formation and regulation of mitochondrial membranes. *Int. J. Cell Bio.* **2014**: 709828.13. https://doi.org/10.1155/2014/709828.

40. Ferreira Cda S, Maganhin CC, Simões Rdos S, Girão MJ, Baracat EC (2010) Melatonin: cell death modulator. *Rev. Assoc. Med. Bras*. **56** (6): 715-718.

41. Wang LM, Suthana NA, Chaudhury D, Weaver DR, Colwell CS (2005) Melatonin inhibits hippocampal long-term potentiation. *Eur. J. Neurosci.* **22** (9): 2231–2237. doi:10.1111/j.1460-9568.2005.04408.

42. Cai J, Yang J, Dean P (1998) Mitochondrial control of apoptosis: the role of cytochrome C. *BBA*. **1366** (1–2): 139-149.

43. Cheng M, Huang H, Lin Y *et al*. (2019) BA6 Induces Apoptosis via Stimulation of Reactive Oxygen Species and Inhibition of Oxidative Phosphorylation in Human Lung Cancer Cells. *Oxid. Med. Cell Longev.* **2019**: 6342104. https://doi.org/10.1155/2019/6342104.

44. Wang X (2009) The antiapoptotic activity of melatonin in neurodegenerative diseases *CNS Neurosci. Ther.* **15** (4): 345-357.

45. Vakifahmetoglu-Norberg H, Ouchida AT, Norberg (2017) The role of mitochondria in metabolism and cell death. *BBRC*. **482** (3): 426-431. doi: 10.1016/j.bbrc.2016.11.088.

46. Alkalby JM, Sarah JS (2013) Effect of propylthiouracil-induced hypothyroidism on reproductive efficiency of adult male rats. *Bas. J. Vet. Res*. **12** (2): 113-121.

47. Hidayat M (2012) Protective role of melatonin and insulin on streptozotocin induced nephrotoxicity in albino rats. *Pak. J. Med.& Health Sci.* **6** (3): 669-674.

48. Hua YC, Chunga MH, Lee TH (2018) An assay of optimal cytochrome c oxidase activity in fish gills. *Anal. Biochem*. **553**: 38-45. doi: 10.1016/j.ab.2018.05.017.

49. Makino Y, Ichimura M (2007) Cytochrome C oxidase as a cause of variation in oxygen uptake rates among vegetables*. J. Am. Soci. Horticul. Sci*. **132** (2): 239-245.

50. Melendez-Ferro M, Rice MW, Roberts RC, Perez-Costas E (2013) An accurate method for the quantification of cytochrome C oxidase in tissue sections. *J. Neurosci. Methods* **214** (2): 156–162. doi:10.1016/j.jneumeth.2013.01.010.

51. Vilhjálmsdóttir J, Gennis RB (2018) The electron distribution in the "activated" state of cytochrome c oxidase. *Sci. Rep.* **8**: 7502.doi:10.1038/s41598-018-25779.

52. Tait SW, Green DR (2013) Mitochondrial regulation of cell death. *Cold Spring Harb. Perspect. Biol.* **5** (9): a008706. doi:10.1101/cshperspect.a008706.

53. Kotiadis VN, Duchen MR, Osellame LD (2014) Mitochondrial quality control and communications with the nucleus are important in maintaining mitochondrial function and cell health. *BBA*. **1840** (4):1254-1265.

54. Chipuk JE, Bouchier-Hayes L, Green DR (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ.* **13**: 1396–1402.

55. Kaniak-Golik A, Skoneczna A (2015) Mitochondria–nucleus network for genome stability. *Free Radic. Biol. Med*. **82**: 73-104.

56. Dowling AL, Martz GU, Leonard JL, Zoeller RT. (2000) Acute changes in maternal thyroid hormone induce rapid and transient changes in gene expression in fetal rat brain, *J. Neurosci.* **20** (6): 2255–2265.

57. Martín M, Macías M, Escames G, Reiter RJ (2000) Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. *J. Pineal Res*. **28**: 242–248. doi: 10.1034/j.1600-079X.2000.280407.

58. Ahmed RG (2012). Maternal-fetal thyroid interactions, *Thyroid Hormone*, N.K. Agrawal (Ed.), ISBN: 978-953-51-0678-4. pp. 125-156. DOI: 10.5772/48076.

59. Stepien BK, Huttner WB (2019) Transport, metabolism, and function of thyroid hormones in the developing mammalian brain. *Front. Endocrinol.* (Lausanne). **10:** 209. doi:10.3389/fendo.2019.00209.

60. Mogulkoc R, Baltaci AK (2003) The effect of intraperitoneal melatonin supplementation on the release of thyroid hormones and testosterone in rats with hyperthyroid. *Neuro. Endocrinol. Lett.* **24** (5): 345-347.

61. Suofu Y, Li W, Jean-Alphonse FG *et al*. (2017) Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proc. Natl. Acad. Sci*. **114** (38): E7997-E8006. doi: 10.1073/pnas.1705768114.

62. Rubenstein JL (2011) Annual research review: Development of the cerebral cortex: implications for neurodevelopmental disorders. *J. Child Psychol. Psychiatry* **52** (4): 339–355. doi:10.1111/j.1469-7610.2010.02307.x.

63. Prezioso G, Giannini C, Chiarelli F (2018) Effect of thyroid hormones on neurons and neurodevelopment. *Horm. Res. Paediatr.* **90**: 73–81. https://doi.org/10.1159/000492129.

64. Patel J, Landers K, Li H, Mortimer RH, Richard K (2011) Thyroid hormones and fetal neurological development. *J. Endocrinol*. **209** (1): 1–8. doi: https://doi.org/10.1530/JOE-10-0444.

65. Rovet JF (2014) The role of thyroid hormones for brain development and cognitive function. Szinnai G (ed): *Endocr. Dev.* **26**: 26-43. doi:10.1159/000363153.

66. Zoeller TR, Dowling AL, Herzig CT, Iannacone EA, Gauger KJ, Bansal R (2002) Thyroid hormone, brain development, and the environment. *Environ. Health Perspect.* **110:** (3): 355–361. doi:10.1289/ehp.02110s3355.

67. D'Angelo G, Marseglia L, Manti S *et al*. (2016) Atopy and autoimmune thyroid diseases: melatonin can be useful? *Ital. J. Pediatr.* **42** (1): 95. doi:10.1186/s13052-016-0305-0.

68. Marin G, Santos F, Bernal M, Martinez G, Roman V *et al*. (2015). Melatonin in the thyroid gland: Regulation by thyroid-stimulating hormone and role in thyroglobulin gene expression**.** *J. Physiol. Pharmacol.* **66** (5): 643-652.

69. Zhang Y, Zhang WX, Zhang YJ *et al*. (2018) Melatonin for the treatment of spinal cord injury. *Neural Regen. Res*. **13** (10):1685–1692. doi:10.4103/1673-5374.238603.

70. Anderson G, and Mazzoccoli G (2019) Left ventricular hypertrophy: roles of mitochondria CYP1B1 and melatonergic pathways in co-ordinating wider pathophysiology. *Int. J. Mol. Sci.* **20** (16): 4068.

71. Musshoff U, Riewenherm D, Berger E, Fauteck JD, Speckmann EJ (2002) Melatonin receptors in rat hippocampus: molecular and functional investigations. *Hippocampus* **12** (2): 165-173.

72. Graziella A, Carlo VL, Ragazzi E, Steinhart H, Varesio L (2003) Developments in tryptophan and serotonin metabolism *Springer-Verlag US.* https://doi.org/10.1007/978-1-4615-0135-0.

73. Lacoste B, Angeloni D, Dominguez-Lopez S, Calderoni S *et al*. (2015) *J. Pineal Res*. **58**: 397–417 doi:10.1111/jpi.12224.

74. Valdés-Tovar M, Estrada-Reyes R, Solís-Chagoyán H *et al*. (2018) Circadian modulation of neuroplasticity by melatonin: a target in the treatment of depression. *Br. J. Pharmacol*. **175** (16): 3200–3208. doi:10.1111/bph.14197.

75. Tan DX, Manchester LC, Reiter RJ, Qi W, Kim SJ, El-Sokkary GH (1998) Melatonin protects hippocampal neurons *in vivo* against kainic acid-induced damage in mice. *J. Neurosci. Res.* **54** (3): 382-389.

76. Zhu Y, Li M, Wang X *et al*. (2011) Caspase cleavage of cytochrome c1 disrupts mitochondrial function and enhances cytochrome c release. *Cell Res*. **22** (1):127-141.

77. Farooqui AA, Ong WY, Horrocks LA (2008) Possible mechanisms of neural injury caused by glutamate and its receptors. *In: Neurochemical Aspects of Excitotoxicity*. Springer, NY.

78. Mansouri A, Demeilliers C, Amsellem S, Pessayre D, Fromenty B (2001) Acute ethanol administration oxidatively damages and depletes mitochondrial DNA in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. *J. Pharmacol. Exp. Ther.* **298**: 737-743.

79. Jou MJ (2011) Melatonin preserves the transient mitochondrial permeability transition for protection during mitochondrial Ca(2+) stress in astrocyte. *J. Pineal. Res*. **50** (4): 427-435. doi: 10.1111/j.1600-079X.2011.00861.

80. Tiong YL, Ng KY, Koh RY, Ponnudurai G, Chye SM (2019). Melatonin prevents oxidative stress-induced mitochondrial dysfunction and apoptosis in high glucose-treated schwann cells via upregulation of Bcl2, NF-κB, mTOR, Wnt signalling pathways. *Antioxidants (Basel)* **8** (7): 198. doi:10.3390/antiox8070198.

81. Perdomo J, Cabrera J, Estévez F, Loro J, Reiter RJ, Quintana J (2013) Melatonin induces apoptosis through a caspase-dependent but reactive oxygen species-independent mechanism in human leukemia Molt-3 cells. *J. Pineal Res*. **55** (2): 195-206. doi: 10.1111/jpi.12062.

82. Kakkar P, Singh BK (2007) Mitochondria: A hub of redox activities and cellular distress control. *Mol. Cell Biochem.* **305** (1-2): 235-253. doi: 10.1007/s11010-007-9520-8.

83. Baghcheghi Y, Salmani H, Beheshti F, Hosseini M (2017) Contribution of brain tissue oxidative damage in hypothyroidism-associated learning and memory impairments. *Adv. Biomed. Res*. **6**: 59.doi:10.4103/2277-9175.206699.

84. Habib S, Ali A (2011) Biochemistry of nitric oxide. *Ind. J. Clin. Biochem*. **26** (1): 3-17.

85. Turrens JF (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol*. **552** (2): 335-344.

86. Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev*. **87** (1): 315–424. doi:10.1152/physrev.00029.2006.

87. Guo C, Sun L, Chen X, Zhang D (2013) Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural Regen. Res*. **8** (21): 2003–2014. doi:10.3969/j.issn.1673-5374.2013.21.009.

88. Nita M, Grzybowski A (2016) The Role of the reactive oxygen species and oxidative stress in the patho mechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxid. Med. Cell. Longev*. **2016**: 3164734. doi:10.1155/2016/3164734.

89. Panel M, Ghaleh B, Morin D (2018) Mitochondria and aging: A role for the mitochondrial transition pore? *Aging Cell* **17** (4): e12793. doi:10.1111/acel.12793.

90. Šileikytė J, Forte M (2019) The Mitochondrial Permeability Transition in Mitochondrial Disorders. *Oxid. Med. Cell. Longev.* **2019**: 3403075. doi:10.1155/2019/3403075.

91. Görlach A, Bertram K, Hudecova S, Krizanova O (2015) Calcium and ROS: A mutual interplay. *Redox Biol*. **6**: 260–271. doi:10.1016/j.redox.2015.08.010.

92. Fischer TW, Zmijewski MA, Wortsman J, Slominski A (2007) Melatonin maintains mitochondrial membrane potential and attenuates activation of initiator (casp-9) and effector caspases (casp-3/casp-7) and PARP in UVR-exposed HaCaT keratinocytes. *J. Pineal Res.* **44** (4): 397-407.

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