1. INTRODUCTION

Extra-pineal melatonin synthesis is not a novelty; many cells and tissues from the immune system have and/or produce melatonin. This is the case in the thymus, which has been described as a tissue with high quantities of melatonin [1]. Its biosynthesis from the amino acid tryptophan requires four enzymatically catalyzed steps [2] that are performed by tryptophan hydroxylase (TPH), aromatic amino acid decarboxylase (AADC), arylalkylamine N-acetyltransferase (AANAT), and hydroxyindole-O-methyltransferase (HIOMT), which is now called N-acetylserotonin-O-methyltransferase (ASMT). The last two steps and hydroxyindole-O-methyltransferase (HIOMT), which is now called N-acetylserotonin-O-methyltransferase (ASMT), were analyzed in the human thymus from children from three different age groups (from days to years). The melatonin membrane and nuclear receptor expression levels also were studied.

2. METHODS

Quantitative reverse transcriptase PCR and western blot were performed to investigate the receptor and enzyme expression levels. The results were examined and correlated with the ages of the thymuses.

3. RESULTS

We found high levels of indolamine in the thymuses of newborns (younger than 1 month), which decreased during development; thymuses from the months (from 2 to 11 months) and years (from 1 to 12 years) groups showed lower levels. A similar decline was also observed in the mRNA of the AANAT enzyme and the expression levels of melatonin receptors. However, ASMT expression was exactly the opposite, with low levels in the newborn group and higher levels in the years group. Our results show that the thymic synthesis of melatonin occurs very early in childhood. Additionally, this is the first report that is focused on melatonin receptors expression in the human thymus.

4. CONCLUSION

Considering the limited melatonin synthesis performed by the newborn pineal gland, we suggest that the high levels of melatonin found in human thymus in this experimental group arise from synthesis in the tissue itself, which could be contributing to the immune efficiency at the thymic level.

Keywords: Melatonin; Thymus; AANAT; ASMT; Melatonin receptor; Nuclear receptor ROR-alpha

Received June 14, 2019 • Revision received July 12, 2019 • Accepted July 21, 2019 • Available online 24 July 2019

https://doi.org/10.1016/j.molmet.2019.07.007
rhythms associated with melatonin present in breast milk. After puberty, melatonin concentrations show a marked nocturnal drop that gradually continue throughout life; melatonin concentrations appear to be severely attenuated but not absent in centenarian individuals [8]. Thus, pineal and plasma melatonin levels fall with advanced age; however, little is known about indolamine levels in extra-pineal tissues throughout life. In the case of the thymus, most of the work performed in rodents indicates that aging decreases the production of melatonin, although in a study performed by our colleagues [9], they hypothesized that there is some resistance by the thymus to maintain its antioxidant capacity. In humans, there was an interesting study performed in elderly people that showed a decrease in melatonin and AANAT levels in very old participants [10]. However, there is no evidence regarding what happens to the melatoninergic system in the thymus during childhood.

Many of the physiological actions of melatonin have been described to be performed through binding to nuclear and membrane receptors, as well as through receptor-independent pathways. The membrane receptors are called MT1 and MT2, and they are part of the G protein-coupled receptor family [11]. However, the recent demonstration of a fully functional mitochondrial GPCR signaling pathway activated by melatonin in the brain [12] leads us to reconsider that G protein-coupled receptors are not only associated with the plasma membrane. Among the nuclear binding sites for melatonin, also known as ROR orphan receptors, three subtypes (α, β, γ) and four splicing variants of the α-subtype are included [13]. This receptor’s family has already been characterized in the thymus from several species, including mouse [14] and rat [15,16]; at present, there is no evidence of melatonin binding sites in the thymus of humans or how they are influenced by age.

To assess whether the thymic melatoninergic system shows changes related to development, we studied the enzyme expression required for melatonin synthesis as well as the nuclear and membrane melatonin receptors in normal human thymuses from children of different ages. The results were correlated with the ages of the tissues. Here, we determined both the expression of the enzymatic machinery for the local production of melatonin as well as the expression of its specific receptors in all studied ages.

2. MATERIALS AND METHODS

2.1. Thymus samples
Thymic tissues from newborns to 12-year-old children were obtained after cardiac surgery procedures (congenital heart diseases). The thymus fragments that made access to the heart more difficult during surgery were removed, rinsed in normal saline solution and frozen at −80 °C until RNA and protein extraction was performed. The study followed the Helsinki Declaration for medical research involving human subjects. Legal representatives of the patients signed the written informed consent to participate in the research. The study was approved by the Ethics Committee of the Virgen del Rocio Hospital in Seville, Spain, on February 11th of 2010 (Act n° 03/2010). The sixty-one (61) samples used in this study were grouped into the following 3 groups of patients: newborns or days (younger than 1 month), months (younger than 1 year), and years (older than 1 year). All the samples’ characteristics are described in Table S1.

2.2. Measurement of thymus melatonin levels
The tissues were weighed, homogenized in PBS, and centrifuged at 3000 g for 10 min; the supernatants (500 μL) were kept for melatonin determination. The melatonin quantity in the tissue was estimated using an ELISA kit (IBL, Hamburg, Germany) according to the manufacturer’s recommendations.

2.3. RNA isolation, reverse transcription, and real-time PCR
RNA was extracted from the organs using the TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Single-strand cDNA was synthesized from 3 μg of RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Real-time PCR was performed on a LightCycler® 480 (Roche) using the LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). The primer sequences are detailed in Table S2. All PCR reactions included negative controls in which the template cDNA was omitted. The expression level of each gene was normalized to that of β-actin, and the relative gene expression was calculated using the 2−ΔΔCt method.

2.4. Western blot
The tissue was homogenized and lysed at 4 °C in lysis buffer containing 50 mM Tris, pH 8; 137 mM NaCl; 10% glycerol; and 40% Nonidet with protease inhibitor cocktail (Sigma—Aldrich, St. Louis, MO, USA). The protein content of the lysates was quantified using the Bradford method [17]. Aliquots containing 75 μg of protein were denatured at 85 °C for 5 min in Laemmli’s Buffer (Sigma—Aldrich), subjected to SDS—PAGE and transferred to a PVDF membrane. The membranes were blocked with Tris-buffered saline—0.05% Tween 20 (TBST) containing 5% nonfat dry milk for 1 h at RT. The blots were then incubated overnight with primary antibodies against human MT1 (Mel1aR, N-20, sc13179) [Santa Cruz Biotechnology, Santa Cruz, CA] at a dilution of 1:200, human MT2 (Mel1bR, G-20, sc-28453) [Santa Cruz Biotechnology, Santa Cruz, CA] at a dilution of 1:200, and human HIOMT (anti-ASMT/HIOMT, LS-C156543) [LSBio] at a dilution of 1:500. The blots were then washed 3 times with TBST before incubation for 1 h with secondary antibodies linked to horseradish peroxidase (anti-Rabbit IgG HRP W401; anti-Mouse IgG HRP W402; and anti-Goat IgG HRP V8051) (Promega). The bound horseradish peroxidase was visualized using the Western Blotting Luminol Reagent sc-2048 (Santa Cruz Biotechnology, Santa Cruz, CA). The bands obtained in the blots were scanned and analyzed using a ChemiDoc- It Imaging System. The amount of protein loaded in each lane was controlled by immunoblotting with the monoclonal anti-GAPDH antibody MAB374 (Millipore) at a dilution of 1:1000.

2.5. Statistical analysis
All results are reported as the mean ± SEM. Data were analyzed with the SPSS® v24.0 software. Kruskal—Wallis test was used to determine the overall differences between groups. Spearman correlation coefficient was used to explore possible associations between variables. Values of p ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1. Melatonin content in the human thymus is higher in newborns
Circulating melatonin levels in the blood of mammals reaches concentrations up to 0.5 nM [18], while extra-pineal concentrations vary depending on the tissue. To determine the melatonin contents in the thymus samples from children and to investigate whether these change with age, indolamine levels were measured in tissues from the three age groups (Figure 1). The highest melatonin levels were found in the thymuses from newborns (approximately 40 pg/mg of tissue). This concentration decreased by half in the thymuses from children under 1
year old, although statistically significant differences were only found between the days and years groups (p ≤ 0.05).

3.2. The expression of the melatonin biosynthetic machinery in the human thymus from children changes with age

Melatonin synthesis is derived from the amino acid tryptophan and is usually found in tissues with AANAT and ASMT activity [2]. To determine the expression of AANAT and ASMT mRNAs in human thymus from children, cDNAs from the three groups of patients (days, months and years) were subjected to real-time quantitative PCR. The assay revealed that AANAT mRNA expression was significantly downregulated with age, with the highest levels of this transcript being measured in the group of neonates (Figure 2A). This decrease was also supported by the negative correlation (ρ = −0.4989; p ≤ 0.001) between AANAT expression and patient age (Figure 2B). In contrast, a significant increase in ASMT mRNA expression was detected in the thymuses of patients older than 1 year (Figure 3A) when compared with the 12 months-younger patients (p ≤ 0.05). This increase was also observed at the translational level (Figure 3B). In western blots, a well-defined band at approximately 52 kDa corresponding to ASMT was found in the three age groups, but the expression was higher in the eldest thymuses when compared with the two other groups (p ≤ 0.001).

3.3. The thymus not only synthesizes melatonin but also responds to it

The transcriptional and translational expression of melatonin receptors was determined using qPCR and western blot analysis, respectively. The results revealed that mRNAs of the MT1, MT2 (Figure 4A1 and B1), and ROR/RZR receptors (Figure 5) were significantly reduced (more than 60%) in the thymuses from the months and years groups on comparison to newborns. These decreases were also observed at translational level for the membrane receptors (Figure 4A2 and B2), but these results were only statistically significant for subtype 1 (MT1).

3.4. The melatonin effector system decreases throughout childhood

The variation in mRNA levels for melatonin receptors throughout childhood was supported by statistical analyses using the nonparametric Spearman r test, which showed a negative correlation between melatonin receptor mRNA expression and the age of the thymus (Figure 6). The membrane receptors showed a strong negative correlation (ρ ≤ 0.001, Figure 6A,B), whereas in the case of nuclear receptors RORα1 and RORα4, the negative correlation was moderate but also significant (ρ ≤ 0.01 and ρ ≤ 0.001, respectively, Figure 6C,D), indicating that expression of the melatonin effector system is higher in newborns and decreases throughout childhood. Additionally, to explore whether this expression could also be associated with the melatonin contents in the thymus, we calculated the correlation coefficients; accordingly, only RORA1 and RORA4 expressions were slightly correlated (ρ ≤ 0.05) with intra-thymic melatonin levels (Figure 7).

3.5. The expression of genes associated with melatonin synthesis and signaling correlate in the human thymus

The dynamic changes in the melatoninergic system in the natural course of development were analyzed again using the nonparametric Spearman r test (Table 1), which showed strong correlations (ρ > 0.8, p ≤ 0.001) between MT1, MT2, and AANAT gene expression. This correlation was also positive and statistically significant between the nuclear receptors RORA1 and RORA4 (ρ = 0.642, p ≤ 0.001). Additionally, the nuclear receptors also showed a moderate correlation that was significant with AANAT, MT1, and MT2.
Figure 3: ASMT is upregulated in the thymus of children. A) ASMT mRNA expression on thymus samples from newborn (named “days”, white bar), younger than 1-year-old (named “months”, gray bar) and younger than 12-year-old (named “years”, black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta C_T}$ equation was applied to calculate the relative expression; the values were normalized to β-actin expression. The data represent the mean ± SEM (n = 48). *p ≤ 0.05. B) Protein extracts from the thymus were analyzed by western blot for ASMT expression at several ages using GAPDH as a loading control. The quantified results (on the top) are expressed as the optical density of the ASMT signal after normalization to GAPDH (bar graph). The images on the bottom are representative of five independent experiments. ***p ≤ 0.001.

Figure 4: Expression of membrane melatonin receptors in human thymus during childhood. MT1 (A1) and MT2 (B1) mRNA expression in thymus samples from newborn (white bar), younger than 1-year-old (gray bar) and younger than 12-year-old (black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta C_T}$ equation was applied to calculate the relative expression; the values were normalized to B-ACTIN expression. The data represent the mean ± SEM (n = 46). *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001. Protein extracts from the thymus were analyzed by western blot for the expression of MT1 (A2) and MT2 (B2) at several ages using GAPDH as a loading control. The optical densities of the signal from both receptors after normalization to GAPDH are shown on the top. The images on the bottom are representative of five independent experiments. ***p ≤ 0.001.
expression. Interestingly, only the RORA1 subtype was positively correlated with ASMT expression ($p \leq 0.05$).

4. DISCUSSION

The leading site for T-cell development in the human body is the thymus; consequently, this is one of the organs involved in the generation and preservation of the adaptive immune system [19]. In terms of the main cellular subpopulations, the human thymus develops fully before birth and begins to atrophy after puberty, when the number of thymocytes decreases and the thymic stroma is gradually replaced by adipose fat throughout a lifetime. However, according to evidence, residual T lymphopoiesis continues until adulthood [20]. It is well-documented that melatonin influences both the morphology and function of this vital organ. The first study that showed a connection between the thymus and melatonin was performed almost 50 years ago, when a pinealectomy caused a reduction in the size of the thymus from 130 to 70 mg in mice [21]. In 1975, other authors also showed a loss in the proliferation of thymic cells after pinealectomy [22]. However, several studies have shown that the age-related thymic involution can be delayed or even reverted. In this sense, the grafting of a pineal gland from young animals into an old one or the administration of melatonin (the pineal secretory product) maintained thymus function and cellularity [23,24]. Moreover, indolamine improves the proliferative

Figure 5: The human thymus expresses nuclear melatonin receptors. RORA1 (A) and RORA4 (B) mRNA expression in thymus samples from newborn (white bar), younger than 1-year-old (gray bar) and younger than 12-year-old (black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression; the values were normalized to β-actin expression. The data represent the mean ± SEM ($n=60$). **$p \leq 0.01$ and ***$p \leq 0.001$.

Figure 6: The melatonin receptor system correlates inversely with age. Correlation analysis between age (days) and mRNA expression ($2^{-\Delta\Delta Ct}$) for MT1 (A), MT2 (B), RORA1 (C), and RORA4 (D) in the human thymus. Each dot represents one thymus, and the straight-line represents the best-fit line obtained by linear regression analysis. ($n=46$ for MT1 and MT2; $n=58$ for RORA1; and $n=60$ for RORA4).
Spearman year of age[29]. These
According to the
life, coinciding with the decrease in thymic epithelial space[28]. In
function gradually decreases in thymopoietic function as from year 1 of
activity of the AANAT and ASMT enzymes in human thymuses in adults
detected great quantities of melatonin as well as the expression and
ence of melatonin receptors in this immune organ from mice and rats
between the thymus and melatonin, which is reinforced by the pres-
minimal for the
synthesis. Taking into account that pineal melatonin synthesis is
youngest age group, it is logical to think that its main source is local
based on the high levels of both enzymes in the thymuses from the
cannot rule out that part of this melatonin comes from the circulation,
to 19 days of age) as well as high levels of melatonin. Although we
expressed from the beginning of birth. We detected the mRNA and
the melatonin biosynthetic machinery in the human thymus is highly
expressed from the beginning of birth. We detected the mRNA and protein expression of both enzymes in thymuses in newborns (from 6
to 19 days of age) as well as high levels of melatonin. Although we
cannot rule out that part of this melatonin comes from the circulation,
based on the high levels of both enzymes in the thymuses from the
youngest age group, it is logical to think that its main source is local
synthesis. Taking into account that pineal melatonin synthesis is
minimal for the first six weeks of life [26], the thymus could show a
compensatory increase in melatonin production, as has been previ-
ously shown [16]. The role of higher melatonin production in the human
thymus at early stages might be related with two main functions:
on one hand by improving the morphology and thymic function and, on
the other, by protecting against the oxidative damage in the tissue [27].
According to the first hypothesis, some authors state that the thymic
function gradually decreases in thymopoietic function as from year 1 of
life, coinciding with the decrease in thymic epithelial space [28]. In
fact, a very recent article showed that the thymic index to weight ratio
(cm3/Kg) was largest at early infancy (1–8 weeks) and smallest at 1
year of age [29]. These findings would match with the higher levels of
melatonin found in the youngest thymuses. Furthermore, since new-
borns are especially susceptible to oxidative stress [30], another
possible role of melatonin at such an early age would be its cytoprotec-
tive ability [31]. In fact, the recent evidence about melatonin is
mainly synthesized in the mitochondria from several tissues lead us to
think that the thymus would not be an exception [32], especially if we
consider that in eukaryotes, mitochondria are the major source of
reactive oxygen species (ROS) and they require specific onsite pro-
tection [32]. Another fact to contemplate is the potential effect of
surgery on increasing local melatonin synthesis as part of the acute
inflammatory response [33], which increases TNF and leads to the
transcription and activation of AANAT. According to this, the presence
of melatonin might help to protect thymocytes against an oxidative and
inflammatory damage. However, this effect would not seem explain
the differences found among the three group of ages since all samples
were obtained from the surgical intervention.
AANAT mRNA expression levels decreased over the course of growth,
both in the months (from 2 to 11 months of age) and years (from 1 to 12
years) groups. In fact, the expression of this enzyme is inversely
correlated with the age of the thymuses. In contrast, the expression
pattern for ASMT was exactly the opposite, with the highest levels found
in the years group. The unexpected result of high levels of AANAT and
low levels of ASMT in the newborn samples might suggest that ASMT
would not play an important role in the thymic synthesis of melatonin
during the early days of birth, with AANAT being primarily responsible
for melatonin levels in the organ. As the thymus matures, AANAT levels
decrease, and ASMT levels rise instead. This increase in ASMT
expression could be a mechanism to overcome the decrease in AANAT
so as to maintain sufficient melatonin levels in the organ. Another
possibility, albeit far-fetched, is that the last two enzymes involved in
melatonin synthesis are inversely, such that the ASMT enzyme acts first
and the AANAT afterwards. This alternate pathway has been observed in
plants [34] and it is worthy of further investigation.
We found that melatonin receptor expression supports the effects of
melatonin produced locally. The presence of melatonin receptors in the
thymus of several species had been previously reported [14,16,35].
However, this is the first description of the expression of a melatonin
effector system in human thymus samples. We detected mRNA and
protein expression of both membrane receptors subtypes. The highest
levels were found in the days group but the expression decreased
throughout childhood. The same mRNA expression pattern was
observed for the nuclear receptors (RORα1 and RORα4 variants), while
the highest levels were observed in newborns. Therefore, we found

![Figure 7: Nuclear melatonin receptor expression correlates positively with thymic melatonin contents. Correlation analysis between the melatonin contents and the mRNA expression (2−ΔΔCt) of RORα1 (A) and RORα4 (B) in the human thymus. Each dot represents one thymus, and the straight-line represents the best-fit line obtained by linear regression analysis. (n = 32 for RORα1 and n = 33 for RORα4).](image-url)

Table 1 – Spearman’s rank correlation coefficients between the mRNA expression of melatonin synthesis and signaling genes.

| Genes | AANAT | ASMT | MT1 | MT2 | RORα1 | RORα4 |
|-------|-------|------|-----|-----|-------|-------|
| AANAT | 1     | −0.1383 | 0.8775*** | 0.8892*** | 0.4219** | 0.3751* |
| ASMT  | 1     | −0.0407 | −0.177 | 0.3399* | 0.205 |       |
| MT1   | 1     | 0.8676*** | 0.4318** | 0.4469** |       |       |
| MT2   | 1     | 0.4264** | 0.3434* |       |       |       |
| RORα1 | 1     |       |       |       |       |       |
| RORα4 | 1     |       |       |       |       |       |

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
that the expression of the MT₁, MT₂, and RORα receptors in the thymus decreased with age. In line with this, an age-dependent decrease in MT₁ and MT₂ receptor expression was also reported in several tissues by Sanchez-Hidalgo et al. [15] and later by Hill et al. [36]; they suggested that this reduction led to the early onset of senescence. In our study, the correlation coefficients showed that the expression of membrane receptors is inversely related with the age of the thymuses, but the expression of nuclear receptors appears to also be associated with melatonin levels. Although a correlation does not indicate causality, in this case, it is not illogical to think that melatonin levels may influence the expression of the nuclear receptor that has already been described to directly interact with it so as to exert its transcriptional functions [37]. We were unable to find a relationship between the melatonin content in the thymus and the other genes in the melatoninergic system, which could be due to the reduced sample size or problems with the melatonin quantification technique.

A logical but not less interesting finding from our study was the highly statistically significant association between the expression of the melatonin membrane receptors and AANAT; at lower enzymes levels, there were also lower receptors levels. These receptors are largely responsible for mediating the downstream effects of melatonin [38], and AANAT is the major enzyme in melatonin synthesis [39]. Thus, an increase in local synthesis of melatonin via AANAT during the early age coincides with an increase in the action of melatonin via MT₁ and MT₂ receptors. The expression of the two RORα isoforms studied was also associated with the expression of AANAT and the membrane receptors, however, these correlations were lower. This is not the first time that we suggest interplay between nuclear and melatonin receptors, as this was previously shown in human lymphocytes [40]. However, the way in which these interactions take place in the thymus and how they are regulated are still poorly understood and require further studies.

5. CONCLUSION

The thymic melatoninergic system is present and especially active from the first days of human life; on excluding ASMT, the thymic melatoninergic system is inversely correlated with the age of the human thymus. We ignored whether these variations are related to changes in the composition and cellularity as described by Weerkamp et al. [41], but we believe that the decrease in the receptors themselves as well as the melatonin content might have an altering effect on the architecture of the thymus, consequently affecting the main function of this organ. Consistent with our conclusion, Odinokov and Hamblin suggest that photo-biomodulation can revert age-associated thymic involution through stimulation of extra-pineal synthesis, thus improving the immune function [42]. Although additional studies are necessary to better understand the network between the neuroendocrine and immune systems within the human thymus, our findings suggest that melatonin locally produced may participate in intra-thymic maturation and T-cell differentiation, which leads to the development of cell-mediated immunity in humans.

STATEMENT OF ETHICS

Legal representatives of the patients have given their written informed consent.

FUNDING SOURCES

This study was supported by the Regional Government Ministry of Health (PI-0209-2010), the PAIDI Program from the Andalusian Government (CTS160), and the Andalusian Public Foundation Progress and Health (PI-0485-2014). ICC received a predoctoral fellowship from the FPU program (FPU13/01210). NAS was supported by the Instituto de Salud Carlos III, Ministerio de Economía y Competitividad (RD06/0013/0001 and RD12/0043/0012).
[9] Sanchez-Hidalgo, M., de la Lastra, C.A., Carrascosa-Salmonal, M.P., Narango, M.C., Gomez-Corvera, A., Caballero, B., et al., 2009. Age-related changes in melatonin synthesis in rat extrapineal tissues. Experimental Gerontology 44:328–334.

[10] Paltsev, M.A., Polyakova, V.O., Kvetnoy, I.M., Anderson, G., Kvetnaia, T.V., Linkova, N.S., et al., 2016. Morphofunctional and signaling molecules overlap of the pineal gland and thymus: role and significance in aging. Oncotarget 7:11972–11983.

[11] Jockers, R., Delagrange, P., Dubocovich, M.L., Markus, R.P., Renault, N., Paltsev, M.A., Polyakova, V.O., Kvetnoy, I.M., Anderson, G., Kvetnaia, T.V., 2016. Melatonin production in infants and the impact of prematurity. The Journal of Clinical Endocrinology and Metabolism 75:367–369.

[12] Reiter, R.J., Mayo, J.C., Tan, D.X., Sainz, R.M., Alatorre-Jimenez, M., Qin, L., 2016. Melatonin as an antioxidant: under promises but over delivers. Journal of Pineal Research 61:253–278.

[13] Haynes, B.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[14] Moore, S.E., Fulford, A.J.C., Susseh, F., Nehe, P., Darboe, M.K., Prentice, A.M., 2019. Thymic size is increased by infancy, but not pregnancy, nutritional supplementation in rural Gambian children: a randomized clinical trial. BMC Medicine 17:38.

[15] Back, K., Tain, Y.L., Sheen, J.M., Huang, L.T., 2012. Melatonin utility in neonates and children. Journal of the Formosan Medical Association – Taiwan yi zhi 111:57–66.

[16] Chaiyant, P., Luengtrakoon, K., Wannakamsuk, W., Vichitrarana, V., Klareit, P., Hormdee, D., et al., 2017. Biological functions of melatonin in relation to pathogenesis of oral lichen planus. Medical Hypotheses 104:40–44.

[17] Tan, D.-X., Reiter, R.J., 2019. Mitochondria: the birth place, battle ground and the site of melatonin metabolism in cells. Melatonin Research 2:44–66.

[18] Markus, R.P., Cecon, E., Pires-Lapa, M.A., 2013. Immune-pineal axis: nuclear factor kappaB (NF-kB) mediates the shift in the melatonin source from pinealocytes to immune competent cells. International Journal of Molecular Sciences 14:10979–10997.

[19] Hill, S.M., Cheng, C., Yuan, L., Mao, L., Jockers, R., Dauchy, B., et al., 2013. Age-related decline in melatonin and its MT1 receptor are associated with decreased sensitivity to melatonin and enhanced mammary tumor growth. Current Aging Science 6:125–133.

[20] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[21] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[22] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[23] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[24] Gupta, S., Haldar, C., Ahmad, R., 2015. Photoperiodic regulation of nuclear melatonin receptor RORalpha in lymphoid organs of a tropical rodent Funambulus penanti: role in seasonal oxidative stress. Journal of Photochemistry and Photobiology B Biology 142:141–153.

[25] Hill, S.M., Cheng, C., Yuan, L., Mao, L., Jockers, R., Dauchy, B., et al., 2013. Age-related decline in melatonin and its MT1 receptor are associated with decreased sensitivity to melatonin and enhanced mammary tumor growth. Current Aging Science 6:125–133.

[26] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[27] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[28] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[29] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[30] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[31] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[32] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[33] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[34] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[35] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[36] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[37] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.

[38] Lardone, P.J., Carrillo-Vico, A., Molinero, P., Rubio, A., Guerrero, J.M., 2009. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61:253–278.

[39] Slominski, R.M., Reiter, R.J., Schlabritz-Loutschew, N., Ostrom, R.S., Slominski, A.T., 2012. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and Cellular Endocrinology 351:152–166.

[40] Weerkamp, F., de Haas, E.F., Sempowski, G.D., Wells, A.F., Hale, L.P., 2000. The human thymus during aging. Immunologic Research 22:253–261.