Research Paper

Exogenous melatonin induced disparate changes in pineal karyomorphology and thyroid cytophysiology and T_4 levels in chick (*Gallus domesticus*)

Chattopadhyay R., *Chakraborty S.
Pineal Research Unit, Department of Zoology, University of Calcutta, 35,
Ballygunge Circular Road, Kolkata 700019, INDIA

Abstract

A composite study including pineal and thyroid cell morphology, hormonal analysis and function in response to exogenous melatonin administration awaits in depth investigation in any bird. To investigate the pineal and thyroid responsiveness to exogenous melatonin from combined morphological and hormonal studies in neonatal chicks – two groups, each with eleven chicks were used as control (C) and treated with melatonin (M) at a dose of 100 µg.100 g body weight for fifteen consecutive days. The treatment with melatonin induced stimulation of pineal karyomorphology as evidenced from significantly increased nuclear diameter in the melatonin treated group. On the contrary, melatonin used inhibition of thyroidal activity as evidenced by significantly decreased values of thyroid nuclear diameter, thyrofollicular epithelial height, diameter and D/N activity associated with significantly decreased T_4 values in neonatal male chicks. Our study thus shows that exogenous melatonin induced disparate changes in pineal and thyroid gland activity in these neonatal chicks, whereas pineal gland showed an overall stimulation; in contrast, melatonin induced thorough inhibition of thyroid cytophysiomorphology and hormonal milieu compared to control group of neonatal chick (*Gallus domesticus*)

Introduction

The pineal gland which over the centuries has been associated with an aura of mysticism regarding its alleged function has emancipated and interestingly research on the pineal gland has flourished within past few decades. Over the last few decades the physiological effects of the pineal gland have been investigated primarily in the reference to the reproductive system. This has often led to the tactic assumption that a primary effect of the pineal gland is on the neuroendocrine reproductive axis. Although the photoperiod by way of the pineal gland has obvious effects on certain photoperiodic species, it appears erroneous to consider the pineal as being exclusively or primarily concerned with the reproductive system[1].

It was soon observed that apart from the pineal gland / and melatonin participating in several neural, neuroendocrine and photosexual responsiveness, it virtually controls every hormone secretion by the pituitary gland. The relationship of pineal gland with other peripheral endocrine organs has gradually attracted the scientific community of endocrinologists. Interestingly, apart from the occurrence of melatonin receptor in pineal[2]. The ontogeny of melatonin receptor expression in the thyroid gland has

been best characterized using the Siberian hamster[3-6]. Logistically its interrelationship with one of the most prolific endocrine organ, the thyroid has often raised interest. Information concerning the influence of the pineal gland and its hormonal principle melatonin on thyroid gland is inconclusive in mammals and birds. Morphological data have indicated that the pineal gland in mammals and birds may itself be a target organ for exogenously administered melatonin[7-11]. Ultrastructural studies indicate that the melatonin increases the number of pinealocytes secretory granules, Golgi apparatus, suggesting the activation of the pineal gland [12]. There are however, earlier findings that do not confirm the foregoing observations, indicating that chronic melatonin administration fails to influence the rhythm of melatonin secretion although it moderately increases plasma melatonin levels at night with almost normal level by the following morning [13]. However, collectively, these data point to an acute regulatory action of exogenous melatonin on the pineal melatonin synthetic pathway[14].

Additionally the possibility of melatonin being involved in influencing the pineal self activation has alluded several workers. In rats melatonin has been found to elevate hydroxyindole-*O*-methyl-transferase (HIOMT), serotonin *N*-acetyl transferase (NAT) and melatonin[15-18]. Additionally, *in vitro* study in rat and hamsters indicated that pineal gland is itself a target organ of exogenous melatonin. Melatonin is also implicated in the control of protein/peptide secretion in the pineal gland[19-20].

Ultrastructural studies have also substantiated the above, as it was seen that in rats, exogenous administration of melatonin stimulated the pinealocyte dense cored or granulated vesicles[21] and also, it increases the number of pinealocyte secretory granules and Golgi apparatus suggesting activation of pineal gland[12]. More recent evidences have accumulated from ultrastructural study of rat and mouse which shows that melatonin increases the number of secretory granules of the pinealocytes and participates seledctively upon the secretory mechanism of the pinealocytes[22]. Furthermore, morphological data have affirmed that pineal gland itself may be a target organ for exogenously administrated melatonin[8].

Morphological studies dealing with the influence of melatonin on histological appearance and growth of the thyroid gland have indicated direct effect of this pineal indoleamine at thyrofollicular level suggested that melatonin besides influencing the pineal activity, may also regulate thyroid function[10, 23-25]. Contradiction exists regarding the influence of the pineal gland and melatonin on thyroid activity, ranging from no effect[26] to inhibition[10,25,27-30] or even significant stimulation of thyroxine production by thyroid in mammals[29-34].

Influence of melatonin on the morphology of the thyrofollicular activity in male *Blossomheaded parakeets* (*Psittacula cyanocephala*) and Indian weaver birds (*Ploceus philippinus*) were examined [25]. The findings revealed that exogenous melatonin increased the ratio of follicular diameter to the number of nuclei in these follicles. Melatonin also caused a decrease in the epithelial percentage and epithelial cell height in both species of birds. This implied a suppression of the thyroid gland activity suggestive of an existence of a negative feedback interrelationship between the pineal hormone, melatonin, and the thyroid gland morphology in these two tropical avian species.

In view of the information presented, it is quite apparent that any composite study regarding the influence of exogenous melatonin simultaneously on pineal and thyroid karyomorphology and cell activity along with the measure of changes in thyroxine (T_4) profile appears to have remained undocumented, as of yet, in any avian species. Consequently, with such an endeavour, in the present investigation, we studied the influence of exogenous melatonin on pineal and thyroid karyomorphology, cell activity and thyroidal hormonal changes and function in male neonatal chicks.

Materials and Methods

The pineal-thyroid interrelationship was evaluated following manipulation of the pineal and thyroid gland by administration of melatonin in neonatal male chicks (body weights 70-80 g) acclimatized to laboratory conditions for three days. A total of twenty two chicks were used for the experiment. The animals were divided into two groups A (N=11) control and Group B (N=11) melatonic treated.

Control-Ethanol-Saline vehicle treatment: This included the control group of birds. The chicks (N=11) were injected with vehicular ethanol-saline solution. A daily dose of 0.1 ml vehicle was injected for fifteen days. (Ethanol: Normal saline 1:9 v.v) [8,35].

Melatonin treatment: Comparable to the control group, the chicks (N=11) were injected with melatonin (N-acetyl 1-5 methoxytryptamine, Sigma Chemicals, M.O., USA) dissolved in ethanol: injectable normal saline vehicle (1:9 v/v)[8,35]. The dose of administration being 100 µg/100 gm body weight in 0.1 ml ethanol saline vehicle[36] daily during the entire experimental schedule.

All the injections were given daily subcutaneously in the nape of the neck of the chicks approximately between 16.00 hrs. and 16.30 hrs. because more melatonin binding sites are available near the end of the photophase[13,37]. The duration of the experiment was for fifteen consecutive days. The animals were housed in photoperiodic chambers fitted with lights and exhaust fan. The photoperiodic 12L: 12D lights (on at 06.00 hrs. and off at 18.00 hrs) were controlled by timer switches (Surrey, U.K.). The animals were supplied with standard chick feed and water *ad libitum*. All the experimental animals were maintained and used as per guidelines of Institutional Ethics Committee, University of Calcutta, accredited by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India. After expiry of the experimental period, on the day sixteen (approximately twenty-four hours after the last injection of the drug) each set of control and treated animals were divided into two groups and killed by etherisation. In the first set of control (N=6) and treated (N=6) experiments, animals considered for histological studies. Pineal and thyroid glands were excised and fixed in Bouin's fluid and later processed for paraffin embedding and microtomy. Whereas control (N=5) and treated (N=5) of each species were used for T_4 assay.

Histology

Nuclear size has long been considered to be a reliable index of cellular activity[38] increase in nuclear size has also been considered to reflect an increase in protein content[39-43]. The alteration in the nuclear size influences the synthetic and secretory activity of pineal gland in mammals and birds. The nuclear size responds to various physiologically induced changes and reflects glandular activity in several mammalian and avian species. An active phase is characterized by increased pinealocyte nuclear size indicating stimulation of synthesis activity, whereas an inhibitory phase is characterized by decreased nuclear size suggestive of inhibition of gland cells[8-9, 43-66]. Also, evidences elucidate that thyroid physiology has a regulatory effect on pineal synthetic and secretory activity[59-60, 65-69].

Pineal karyomorphology

In view of the foregoing observations, in the present investigation the pineal gland activity had been mainly judged from karyometric values of the pineal parenchymal cells. Investigations were carried out from 5 µm thick sections stained with iron-alum-haemotoxylin and eosin. Thus for morphometric evaluations at least 250 oval to round nuclei were measured from each of the five randomly selected mid sagittal sections per specimen. Unlike oval nuclei, where the mean of short and long axis were measured, in case of round nuclei only the diameter was measured. In all cases, nuclei were measured under oil immersion using 15 ocular X 100 objective lenses along with ocular micrometer scale. All ocular diameter values were then converted to µm values. Individual values of the specimen were the mean figure of those five sectional measurements. The final mean values of the experimental and control groups were computed form these individual measurements.

Thyroid karyomorphology and histomorphometry

Histological investigations were carried out in the thyroid gland sections (5 µm), which were stained using Ison-alum-haematoxylin and eosin. In addition to histological observations the follicular diameter and the number of nuclei in the same follicle was noted. The ratio of the follicular diameter to the number of nuclei per follicle was evaluated and expressed as D/N value. Additionally, morphometric data of epithelium percentage and cell height of the thyrofollicular cells were recorded. All ocular measurements were converted to µm[25,57].

Hormone Assay

The second set of control (N=5) and treated (N=5) experiments animals were considered for T_4 (3,5,3´,5´-L-tetraiodothyronine) assay, using Automated Chemiluminescence System. The ACS 180 ® according to manufactures protocol. In the circulation 99.95% of T_4 is reversibly bound to transport proteins, primarily thyroxin binding globulin (TBG) and to a lesser extent albumin and prealbumin.

Unbound or free T_4 is metabolically active and bound T_4 is metabolically inactive, acting as a reserve [70-71]. The ACS: 180 T_4 assay is a competitive immunoassay using direct chemiluminescent technology. T_4 in the sample competes with T_4, which is covalently coupled to paramagnetic particles in the Solid Phase, for a limited amount of acridinium ester – labeled monoclonal mouse anti – T_4 antibody in the Light Reagent. The system automatically performs the steps and with the input of Master Curve Card values, the system reports the results according to the selected options as described in systems operating instructions. The system reports T_4 results in µg/dL (mass units) or ηmol/L (S I Units), depending on the units defined when setting up the assay. The ACS: 180 T_4 assay measures T_4 concentrations up to 30 µg/dL (387nmol/L) with a minimum detectable concentration of 0.5 µg/dL (6.4 nmol/L).

Statistical Analysis

Values were presented as the means of the observations following experimental manipulations. All the karyomorphological and biochemical values for the control and treated animals were compared and the level of significance was statistically evaluated by Student's "t" test [72] and through ANOVA (using the package Microcal Origin, Version 4.00).

Results and Discussion

Pineal gland

Control-Ethanol-saline vehicle treatment: Light microscopic studies of chicks pineal gland indicated that the pineal parenchyma was seen to be compact with occasional lobules. The lobules were of irregular size separated from each other by narrow connective tissue strands. These lobules comprised of pinealocytes of principal cells, arranged either of clusters of rosette without lumen or follicles and tubules with lumen interspersed with packed cells. The follicles or tubules contain lumen with periluminal columnar epithelial cells having basal nuclei (Figure 1).
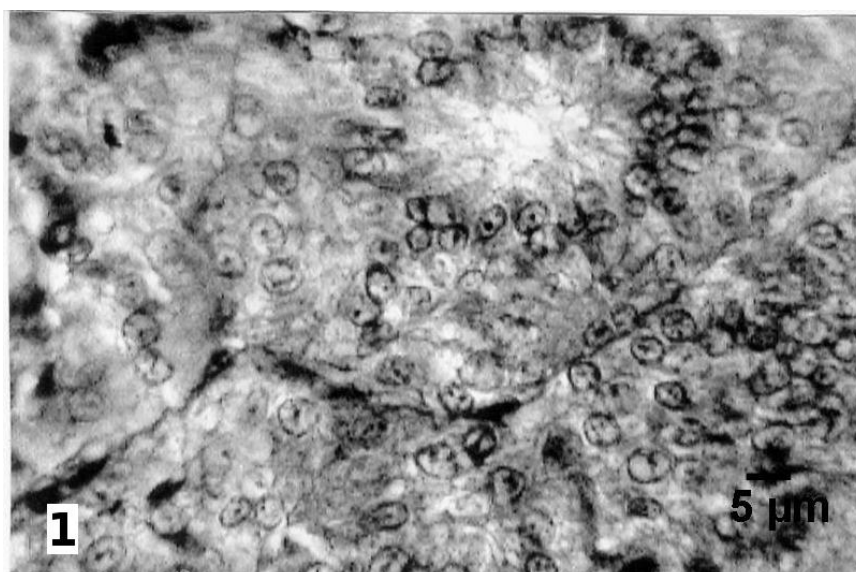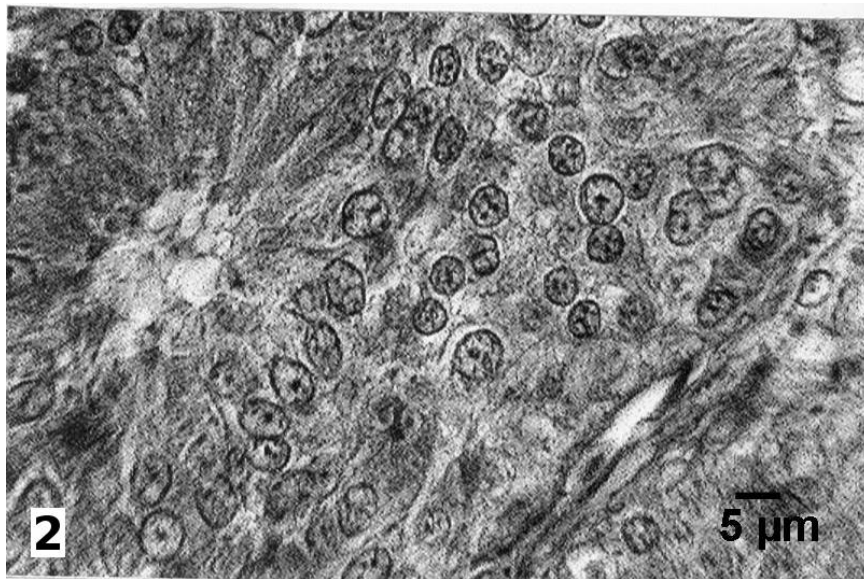

Figure 1: Microphotograph of pineal gland section in control chick showing normal nuclear diameter

The staining procedures used in the present investigation failed to differentiate the parenchymal cell types of pinealocytes. The pinealocytes showed finely granular acidophilic cytoplasm with a spherical, rather vesicular nucleus which often showed presences of a nucleolus. The pinealocytes with distinct round nucleus characterized the pineal gland sections of these vehicular control birds.

Melatonin treatment: Melatonin administration evoked a significant alteration in the pineal indicative of stimulation. The pinealocytes were seen mostly with large round, well defined nuclei (Figure 2). The

values of nuclear diameter of the pinealocytes were increased compared to the control values (p<0.001, Figure1A). The nucleolus appeared to be dense and conspicuous.

Figure 2: Microphotograph of pineal gland section in melatonin treated neonatal chick showing hypertrophied nuclei

The results from one – way analysis of variance for nuclear diameter [F (1,10) = 24.614, p<0.001] of pinealocytes from control and melatonin treated groups revealed that the experimental mean values were significantly different, indicating significant changes between the melatonin treated groups compared with control mean values.
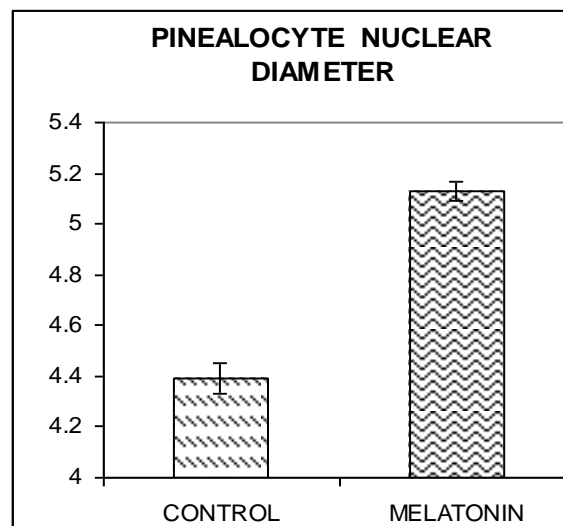
Figure 1(A): Histogram showing pinealocyte nuclear diameter (μm) following melatonin treatment as compared to control values. Melatonin caused a significant increase in nuclear diameter value compared to control group. The vertical lines signify the SEM.
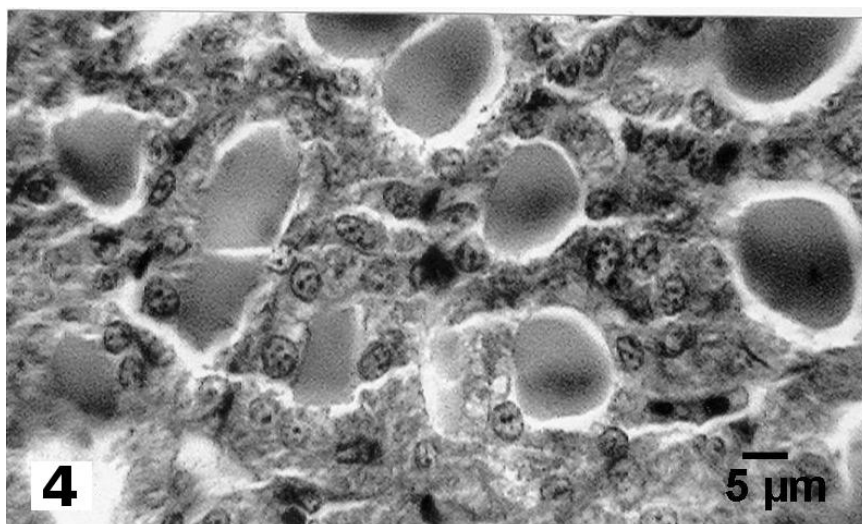
Thyroid gland

Control-Ethanol-saline vehicle treatment: Histological aspect of the control thyroid gland revealed follicles consisting of spheres formed by simple epithelium whose follicular radius was seen to be normal. Follicles were regular in size and shape and the follicle lumina were filled with homogenous colloid (Figure 3).
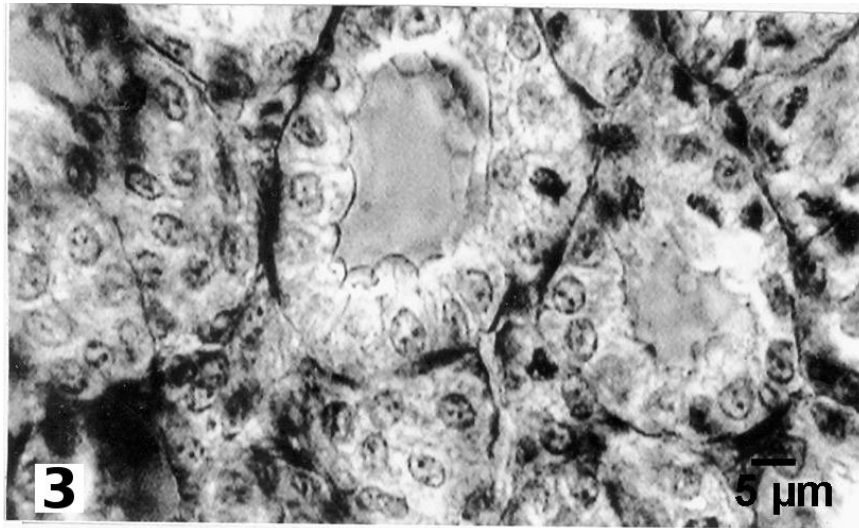
Figure 3: Microphotograph of thyroid gland section in control neonatal chick showing normal nuclear diameter, epithelium height and follicular diameter.

Plasma thyroxine level in these chicks was a moderate value (2.45 µg/dl). The value found in these chicks was within the range as reported earlier. It was seen that in chicks of 1 day 6 weeks old were in the range 1.45-3.0 µg/dl [73].

Melatonin treatment: Melatonin treatment induced significant alteration in the overall histology of the thyroid gland (Figure 4). The epithelial cells were flattened leading to a significantly lower nuclear diameter value (p<0.001, Fig. 1Ba). The thyrofollicular diameter of melatonin treated chicks was somewhat smaller compared to the control group (p<0.001, Figure 1Bb). The epithelium height of thyroid follicles in the melatonin chicks was reduced significantly compared to thyroid of control birds (p<0.001, Figure 1Bc). The D/N value was also less compared to those of control chicks (p<0.01, Figure1Bd).


Figure 4: Microphotograph of thyroid gland section in melatonin treated neonatal chick showing significantly decreased nuclear diameter and decreased epithelium height.

Analysis of variance (one-way) for thyroid nuclear diameter [F(1,10)=15.861, p<0.001], thyrofollicular diameter [F(1,10)=14.935, p<0.001]. D/N [F(1,10)=7.715, p<0.01] epithelial height [F(1,10)=35.315,

p<0.0001] of the thyroid gland from control and melatonin treated animals reveal significant changes of the experimental values compared to the control groups.

The plasma thyroxine level in chicks following melatonin treatment was found to be significantly reduced (1.33 µg/dl) when compared to control group of chicks injected with ethanol-normal saline control vehicle (p<0.001, Figure 1C). One-way analysis of variance of thyroxin content in control and melatonin treated animals [$F_{(1,10)}=132.857$, p<0.001] reveal significant decrease of the mean thyroxin values in the melatonin treated groups compared with the control groups.

THYROID NUCLEAR DIAMETER
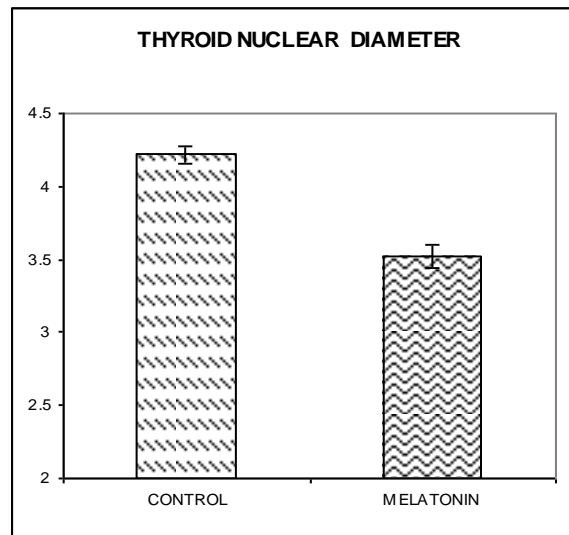
Figure 1 B (a) Histogram showing comparison between control and melatonin treated chicks as regard to thyroid nuclear diameter (µm) value. Melatonin caused a significant decrease in nuclear diameter (µm) value compared to control group. The vertical lines signify the SEM.

THYROID FOLLICULAR DIAMETER
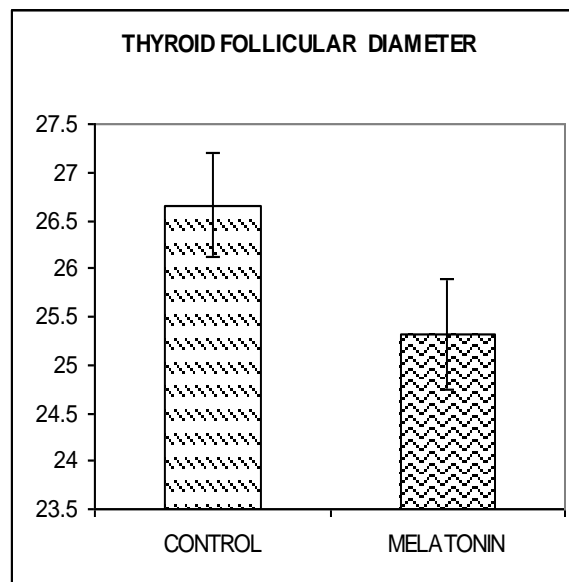
Figure 1 B (b) Histogram showing comparison between control and melatonin treated chicks as regard to thyroid follicular diameter (µm) value. Melatonin failed to produce any significant change in the same. The vertical lines signify the SEM.

THYROID EPITHELIAL HEIGHT
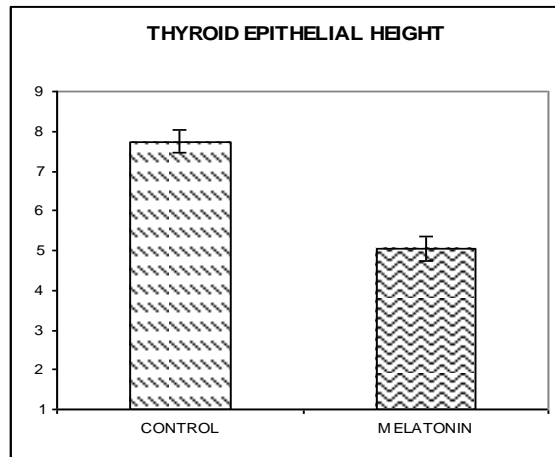
Figure 1 B (c) Histogram showing comparison between control and melatonin treated chicks as regard to thyroid epithelium height (μm) value. Melatonin caused a significant decrease in epithelium height (μm) value compared to control group. The vertical lines signify the SEM.
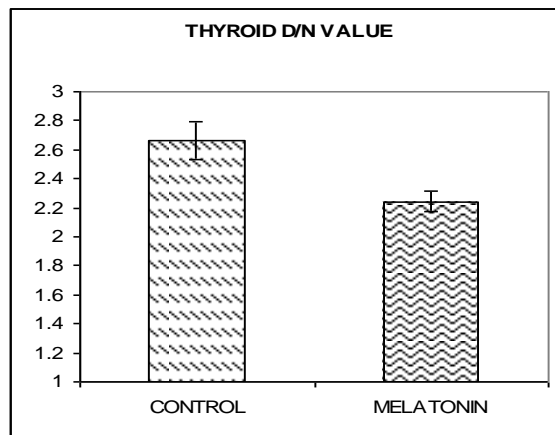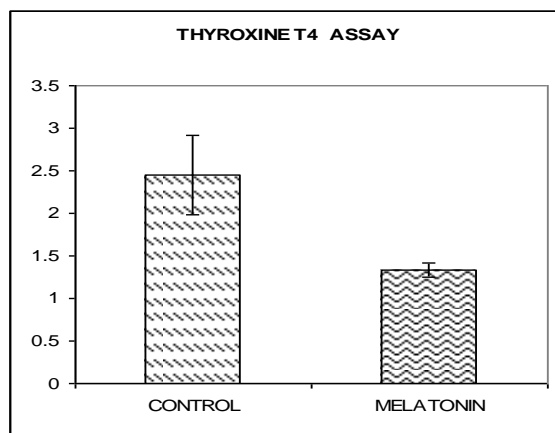

THYROID D/N VALUE

Figure 1 B (d) Histogram showing comparison between control and melatonin treated chicks as regard to thyroid D/N (μm) value. Melatonin caused a significant decrease in D/N (□ m) value compared to control group. The vertical lines signify the SEM.

THYROXINE T4 ASSAY

Figure 1 C Histogram comparing the effect of melatonin treatment on serum thyroxin (T4) content (μg / dl.) with respect to control value. Melatonin caused significant decrease in thyroxin content value as compared to the control group. The vertical lines signify the SEM.

The present investigation offers an insight into the morphological and functional organization of the pineal and thyroid in response to differential modulation of both the glands through administration of pineal indoleamine:melatonin. This study further gives emphasis on the cytophysiological behavior of the glands as reflected in concomitant alterations in the karyomorphological values in both the glands and their possible interrelationship. In addition, estimation of thyroxin content following experimental modulation of both the glands was also performed.

It may be noted that present studies related to the cytological features included measurement of the nuclear diameter, an indicator of the glandular activity[39-42]. The alteration in the nuclear size influences the synthetic and secretory activity of both pineal gland and thyroid in mammals and birds. The nuclear size responds to various physiologically induced changes and reflects glandular activity in several mammalian and avian species. An active phase is characterized by increased pinealocyte nuclear size indicating stimulation of synthesis activity, whereas an inhibitory phase is characterized by decreased nuclear size suggestive of inhibition of gland cells[8,9,43-49].

The current experimental observations reveal that the pineal gland reacts to the indoleamine input by becoming significantly hyperactive, in so far as the synthetic phase of the pineal parenchymal cells are concerned. Melatonin administration in neonatal chicks induce a significant increase in the pinealocyte nuclear diameter with further augmentation of overall picture of the pineal parenchyma as compared to control group of chicks. Thus this quantitative nuclear morphology indicates that in this species, melatonin has a hormonal action on the pineal gland, influencing its metabolism. In fact, the present cytological observations corroborate with earlier biochemical findings in rats where it was observed that melatonin administration caused alteration in the lipid content of the pineal gland and also elevated HIOMT and S-NAT activity, required for melatonin synthesis by the pineal gland [16,17].

Our current studies lend support to the contention that in addition to alterations in pineal biochemistry [8,11,16,74-77] and pineal induced physiological activities[76,77] also seems to serve as a target organ for exogenously administered indoleamine. In addition, apart from the occurrence of melatonin receptor in pineal[27] the result from a later study indicated an acute regulatory action of exogenous melatonin on the pineal melatonin synthesis pathway[14].

Reports about the stimulatory action of the indoleamine on pinealocyte morphology[8,11], pineal ultrastructural components[12] and serotonin secretion[14] confirm our foregoing observations, revealing melatonin to have a stimulatory action on the pineal gland activity. Additionally, in view of the present findings, it is indicated that the pineal gland in neonatal chicks may itself be a target organ for exogenously administered melatonin similar to that observed in mammals[7] and the changes brought about in this species may be a direct action of exogenously administered hormonal impact on the pineal gland.

In fact cytological observation indicating the ability of exogenous melatonin to stimulate pinealocyte morphology and function corroborates with findings by several group of researchers. The fact that pineal gland possesses melatonin receptors[2] confirms the ability of the pinealocytes to respond to melatonin stimulation as evidenced from the current study. Additionally a number of reports substantiate the present findings, in as much as melatonin's ability to stimulate pineal morphological and ultrastructural features[8,11-12,21-22,78] and enhance the biochemical and hormonal activity of the pineal gland[16,17,19,20].

An in-depth study of published literatures indicated that melatonin besides influencing the pineal activity, may also regulate thyroid function[23-25]. Contradiction exists regarding the influence of the pineal gland and melatonin on thyroid activity, ranging from no effect[26] to inhibition[27-30] or even significant stimulation of thyroxine production by thyroid in mammals[29,31,32,34].

The present experimental results show that exogenous melatonin treatment appears to have an inhibitory effect on the thyroidal activity in neonatal chicks. This is characterized by a significant decrease in the thyroid nuclear size in melatonin treated chicks supported by the significant decrease in epithelial height and follicular diameter. With the increase in the number of nuclei all around the epithelium lining the follicular lumen, a decrease in the ratio of follicular diameter to the number of nuclei present in these follicles corroborated suppression of thyrofollicular activity induced by

treatment of melatonin in birds[25]. The biochemical data of the present experimentation further substaintiate the histological observations indicating that melatonin administration significantly depresses the plasma thyroxin content in the neonatal chicks.

These findings are supported by studies, which point to a modulatory depressant effect of the pineal on thyroid gland. Enhancing pineal activity by exposing animals to continuous darkness[28,30] or injecting melatonin[27,29] brought about a generalized depression of thyroid function, whereas abolition of possible pineal influence by its removal[79,80] or inhibition of pineal activity by exposing the animal to continuous light[30,33] or blocking indoleamine synthesis by p-chlorophenylalanine[81], was followed by increased thyroid activity.

The observations on thyroid abnormalities and the earlier results[82-84] suggest that the pineal exerts an inhibitory effect over thyroid function and that the active principle may be melatonin. Our results also corroborate the reports of others where daily injections of melatonin in microgram amounts for several weeks are associated with a depression of plasma thyroxin levels[85]. Additional reports in support of our results[86-88], which indicated that serum T_4 levels are significantly reduced by melatonin administration. A depression in serum T_4, T_3 and TSH levels in female hamsters was reported[87] following melatonin injections for 8 weeks. It was found that serum T_4 levels were lowered by melatonin injections. Hence from a close consideration of earlier reports in mammals compared with the currently obtained experimental results in neonatal chicks regarding the influence of melatonin on pineal and thyroid, it is summarized that the effect of melatonin under similar experimental conditions are that of stimulation of the pineal and inhibition of thyroid as evident from both histological and biochemical evaluations.

Conclusion

On the present study influence of melatonin on pineal and thyroid activities were observed. It was found that although melatonin stimulated the pineal gland activity as evidenced from increased karyomorphological values. On the other hand melatonin induced overall suppression of thyroid gland activity as evidenced from decreased T_4 value and inhibition of thyroid cytomorphometric activity. In essence, the current investigation suggest on disparate effect of melatonin on pineal and thyroid glandular activities in neonatal chicks.

Acknowledgement

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