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# Melatonin and differential effect of L-thyroxine on immune system of Indian tropical bird *Perdicula asiatica*

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**Abstract**

Interaction of thyroxine and melatonin on immune status was noted in vivo and in vitro when peripheral melatonin was high and thyroxine low in plasma of male *Perdicula asiatica* during reproductively inactive phase. During this phase exogenous thyroxine (4 μg/100 g. Bwt./day) and melatonin (25 μg/100 g. Bwt./day) increased immune parameters (spleen weight, total leukocyte count, lymphocyte count, percent stimulation ratio) and increased splenocyte density in spleen. In vitro L-thyroxine ($10^{-6}$ M/ml) supplementation decreased the splenocyte proliferation which was reversed by melatonin (500 pg/ml) supplementation. In vivo L-thyroxine showed immunoenhancing effect while in vitro it decreased the splenocyte proliferation presenting a differential effect. In the absence of internal physiological conditions of the birds, $T_4$ showed a negative effect on splenocytes proliferation in vitro when treated alone. However, melatonin maintained its lymphoproliferative effect under both conditions. Thus, avian splenocyte exposed to different hormonal conditions in vitro might have produced different signal peptides other than in vivo, thereby making the result different.


*Keywords:* Melatonin; L-Thyroxine; Biphasic; Splenocyte; Blastogenic response; In vivo; In vitro

## 1. Introduction

In birds and mammals, the thyroid gland and its hormones have been reported to influence reproduction (Haldar et al., 1992; Maitra et al., 2000, 1996), metabolism (Lewinski, 1990). Thyroxine ($T_4$) has been reported to cause marked enlargement of the thymus and increased peripheral lymphocytes (Hassman et al., 1985) while thyroidectomy resulted in hypoplasia of lymphoid organ (Haldar and Singh, 2001) in mammals. In immunodeficient Snell bag dwarf mice thyroxine markedly increased the nucleated spleen cells, plaque-forming cells, and restored the immunological capacity of the animal (Baroni et al., 1969; Pierpaoli et al., 1969). In contrast, few authors have reported that thyroid hormones have no effects on the immune response either in vivo or in vitro (Weetman et al., 1984), while Gupta et al. (1983) reported that thyroid hormone have immune inhibitory effect in mice.

The pineal gland and its principal neurohormone melatonin affect thyroid function (Lewinski, 1990; Shavali and Haldar, 1998) and lymphatic tissue sizes (Haldar and Singh, 2001) of mammals. Virtually in all cases melatonin was examined for its effects on humoral and cell mediated immunity in mammals and never in any avian species though the pineal-thyroid interrelationship in an avian species, *Perdicula asiatica* has been examined in detail (Haldar and Ghosh, 1988) and an inverse relationship was noted. Further, there is no report available regarding the pineal and thyroid gland modulating immune function in any avian species in general and in a tropical seasonally breeding bird in particular. Therefore, in the present investigation we accessed the interaction of melatonin (which influence reproduction in birds and immunity in mammals) in relation with thyroxine (which is responsible for metabolism,

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reproduction in birds, and T cell maturation in mammals) on immune function of a tropical seasonally breeding bird, the Indian jungle bush quail, *Perdicula asiatica*.

## 2. Materials and methods

All the experiments were conducted in accordance with institutional practice and within the framework of revised animals (Scientific Procedures) Act of 2002 of Govt. of India on Animal Welfare.

Adult male quails were collected from the vicinity of Varanasi (Latitude 25 ° 18'N, Latitude 83 ° 01'E) during reproductively inactive phase (in the month of November). The birds were maintained in aviary exposed to ambient environmental conditions (day length 11L: 13D; temp. 13–22 °C) and were fed with millet seed (*Pennisetum typhoides*) mixed with other food grains (paddy, oat, grass seeds, and small lentils etc.) to match with diet in wild and water ad libitum for 2 weeks and then randomly divided into two sets. The nutritional stress if any, was checked by noting initial and final body weight of the birds during experiment, which was always non-significant.

***Set I.*** Birds were divided into four groups and each groups contained seven young adult male birds. The first groups of bird were injected with normal ethanolic saline (0.01% ethanol) 0.1 mL/day. L-thyroxine ($T_4$, Sigma, St. Louis, USA) at a concentration 2 μg/bird/day (near physiological level dose; dissolved in few drops 0.01 N NaOH and diluted with normal saline) was subcutaneously injected to second groups of bird (Chaturvedi and Thapliyal, 1980). Melatonin (Mel, Sigma, St. Louis, USA) at a concentration of 25 μg/100 g body mass (near physiological level dose; dissolved in few drops of ethanol and diluted with normal saline) was subcutaneously injected to third groups of birds (Singh and Haldar, 2005). Fourth group of birds received both melatonin and thyroxine at 1 h interval. All the administrations were given during evening hours for 30 consecutive days. After 24 h of last injection the birds were sacrificed by decapitation during the dark phase of light/dark cycle.

### 2.1. Hematological parameters

Blood was taken in a WBC pipette and diluted 20 times in Turk's fluid (2.0 ml Glacial acetic acid, 0.1 g mercuric chloride, one drop Aniline, and 0.2 g Gention violet) and the white blood cells counted (no./mm3) in Neubauer's counting chamber (Spencer, USA) under the microscope. Thin film of blood was prepared and stained with Leishman's stain and differential leukocyte (lymphocyte) was counted under oil immersion lens of Leitz MPV3 microscope. Lymphocyte counts (no./mm3) was determined from the total and differential leukocyte count by using the following formula:

$$\text{Lymphocyte Count} = \frac{\text{TLC} \times \text{ Lymphocyte percentage}}{100}.$$

### 2.2. Histological parameters

Spleen was dissected out and fixed in Bouin's fluid for routine histological examination. Paraffin transverse sections of 5 μm thickness were cut and then stained with hematoxylin and eosin. Representative photographs of each group spleen were taken with Leitz microscope under 40× magnification.

### 2.3. Reagent and culture medium for blastogenic response

Tissue culture medium RPMI–1640 and all other chemicals were purchased from Sigma–Aldrich Chemicals, USA. The culture medium was supplemented with 100 μg/ml Streptomycin, 100 U/ml Penicillin, and 10% Fetal calf serum. Spleen was dissected out and processed for preparation of single cell suspensions. Number of cells was adjusted to $1 \times 10^6$ cells/ml in culture medium. Two milliliters of spleen cell suspension of each group were placed in duplicates culture tubes and kept at 37 °C in a 5% $CO_2$ incubator for 72 h. Blastogenic response was measured in terms of [$^3$H]thymidine (BARC, India; specific activity 8.9 Ci/mM) uptake against stimulation by Concanavalin A (Con A; T cell mitogen; SIGMA, USA) of the splenocytes (Pauly and Sokal, 1972).

$$\%\text{SR} = \frac{\text{CPM with Con A}}{\text{CPM without Con A}} \times 100$$

***Set II.*** Twenty birds were kept in ambient environmental conditions to observe in vitro effect of L-thyroxine and melatonin. Four sets of culture were prepared with five replicas in each. First set of culture tubes were supplemented with complete culture media RPMI 1640 only. Second set of culture tubes were supplemented with medium along with melatonin (500 pg/ml). Third set of culture tubes were supplemented with medium along with L-thyroxine ($10^{-6}$ M) and fourth set of culture tubes were supplemented with medium along with melatonin and L-thyroxine both of above concentration (Singh, 2003). Blastogenic response was measured in terms of [$^3$H] thymidine (specific activity 8.9 Ci/mM) uptake against stimulation by Con A of the splenocytes as described in Section 2.3. Reagent and culture medium for blastogenic response.

### 2.4. Statistical analysis

Statistical analysis of the data was performed with one way ANOVA followed by Student-Newman–Keuls' test for parametric data. The differences were considered significant when $P < 0.01$. Kruskal–Wallis test performed for non parametric data. We performed Shapiro–Wilk test to check the normality. Normality was consider when $P > 0.05$.

## 3. Result

### 3.1. Spleen weight

Melatonin treatment significantly ($P < 0.01$) increased spleen weight of birds when compared with control birds

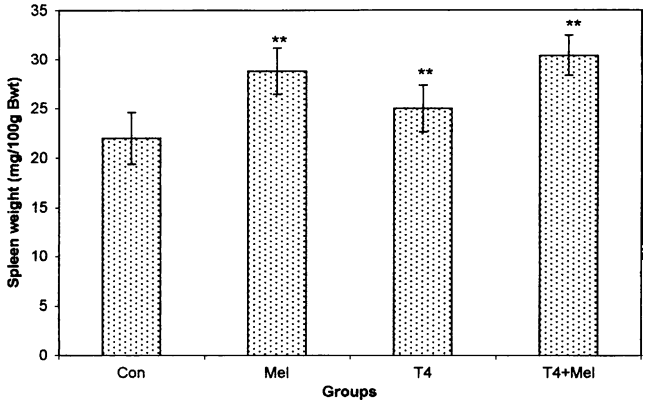


Fig. 1. Effect of L-thyroxine and melatonin administration on spleen weight of Indian jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (November to January). Histograms represent Means ± SE, $n = 5$ for each group within this experiment. Con, control; Mel, melatonin; $T_4$, L-thyroxine. *$P < 0.05$ Con vs $T_4$, **$P < 0.01$ Con vs Mel, Con vs Mel+$T_4$, and $T_4$ vs Mel+$T_4$.

(Fig. 1). A significant ($P < 0.05$) increase was noted in the spleen weight of L-thyroxine treated bird when compared with control birds. L-thyroxine along with melatonin administration caused significant ($P < 0.01$) increase in spleen weight when compared with control birds as well as only L-thyroxine treated birds (Fig. 1; Data parametric, Shapiro–Wilk test, $P < 0.05$, $= 0.7979$; One way ANOVA, $F = 15.08$, dfs: msb = 3, msw = 16 for each group).

### 3.2. Total leukocyte count and lymphocyte count

Melatonin treatment caused significant ($P < 0.01$) increase in total leukocyte count (TLC) (Fig. 2A) and lymphocyte count (LC) when compared with control birds (Fig. 2B). No significant change was noted in TLC of L-thyroxine treated bird, whereas, LC significantly increased ($P < 0.05$) in L-thyroxine treated bird. L-thyroxine treatment along with melatonin to the bird caused significant ($P < 0.01$) increase in total leukocyte count and lymphocyte count when compared with control birds as well as L-thyroxine treated birds (Data parametric, Shapiro–Wilk test, $P < 0.05$, $= 0.3969$ (TLC), $= 0.2214$ (LC); One way ANOVA, $F = 11.9289$ for TLC and $F = 18.022$ for LC, dfs: msb = 3, msw = 16 for each group).

### 3.3. Blastogenic response

#### 3.3.1. In vivo

Melatonin treatment increased significantly ($P < 0.01$) basal and mitogen Con A induced blastogenic response as well as percent stimulation ratio (% SR) when compared with control bird (Fig. 3A). Significant ($P < 0.01$) increase was also noted in basal as well as mitogen Con A induced blastogenic response of splenocytes of L-thyroxine treated bird, but no change was noted in the percent stimulation ratio when compared with control bird. Melatonin treatment along with L-thyroxine significantly increased basal

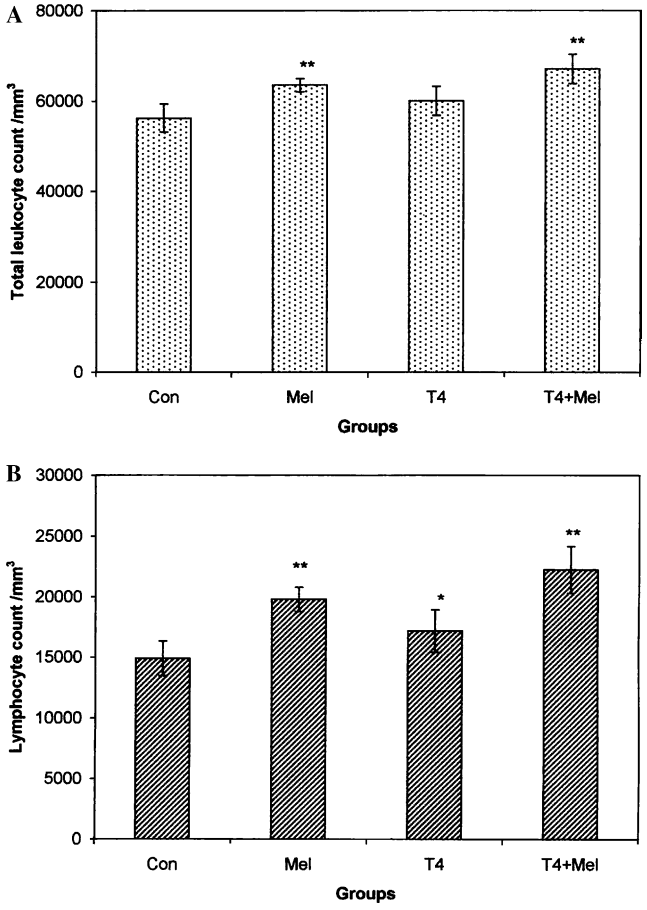


Fig. 2. Effect of L-thyroxine and melatonin administration on (A) total leukocyte count (TLC) and (B) lymphocyte count (LC) of Indian jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (November to January). Histograms represent Means ± SE, $n = 5$ for each group within this experiment. Con, control; Mel, melatonin; $T_4$, L-thyroxine. *$P < 0.05$ Con vs $T_4$, **$P < 0.01$ Con vs Mel, Con vs Mel+$T_4$, and $T_4$ vs Mel+$T_4$.

as well as mitogen Con A induced blastogenic response and percent stimulation ratio when compared with control group as well as L-thyroxine treated birds (Fig. 3B; Data parametric, Shapiro–Wilk test, $P < 0.05$, $= 0.1178$ (Basal), 0.1051 (% SR); One way ANOVA, $F = 150.921$ for basal group, $F = 357.03$ for % SR; dfs: msb = 3, msw = 16 for each group; Non parametric data, Con A supplemented group, Kruskal–Wallis test, $\chi^2 = 17.857$, df = 3, Asymp. Sig. = 000).

#### 3.3.2. In vitro

Melatonin supplemented medium significantly ($P < 0.01$) increased basal and mitogen Con A induced blastogenic response as well as percent stimulation ratio (Fig. 4A). L-thyroxine supplementation in culture significantly ($P < 0.01$) suppressed the basal as well as mitogen Con A induced blastogenic response and percent stimulation ratio. Melatonin supplementation along with L-thyroxine restored the basal blastogenic response as observed in control but significantly ($P < 0.01$) increased the mitogen Con A induced blastogenic response and per-

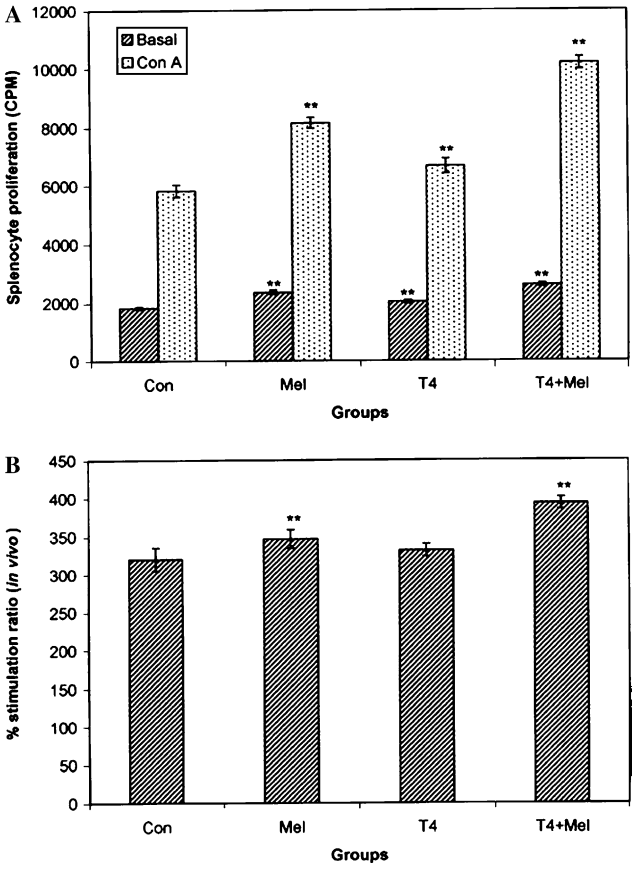


Fig. 3. Effect of L-thyroxine and melatonin administration on (A) basal and mitogen Con A induced blastogenic response in count per minute (CPM) and (B) percent stimulation ratio (% SR) of splenocytes of Indian jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (November to January). Histograms represent means ± SE, $n = 5$ for each group within this experiment. Con, control; Mel, melatonin; $T_4$, L-thyroxine. $^{**}P < 0.05$ Con vs $T_4$, Con vs Mel, Con vs Mel+$T_4$, and $T_4$ vs Mel+$T_4$.

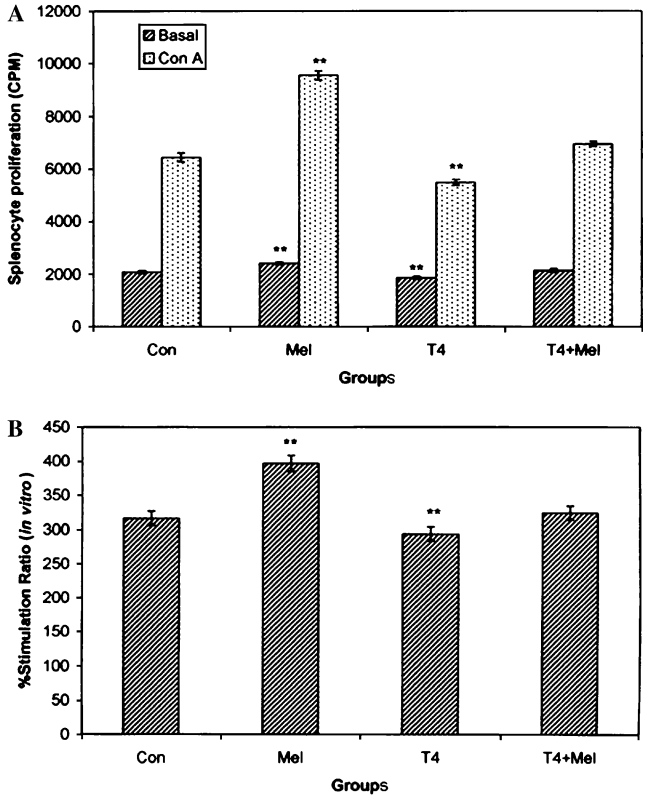


Fig. 4. In vitro effect of L-thyroxine and melatonin supplementation on (A) basal and mitogen Con A induced blastogenic response in count per minute (CPM) and (B) percent stimulation ratio (% SR) of splenocytes of Indian jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (November to January). Histograms represent means ± SE, $n = 5$ for each group within this experiment. Con, control; Mel, melatonin; $T_4$, L-thyroxine. $^{**}P < 0.05$ Con vs $T_4$, Con vs Mel, Con vs Mel+$T_4$, and $T_4$ vs Mel+$T_4$.

cent stimulation ratio when compared with control group (Fig. 4B; Data parametric, Shapiro–Wilk test, $P < 0.05$, = 0.1178 (Basal); One way ANOVA, $F = 73.439$, dfs: msb = 3, msw = 16 for each group; Non parametric data, Con A supplemented group, Kruskal–Wallis test, $\chi^2 = 17.857$, df = 3, Asymp. Sig. = 000; % SR, Kruskal–Wallis test, $\chi^2 = 14.964$, df = 3, Asymp. Sig. = 002).

### 3.4. Changes in spleen histology

Melatonin treatment to bird increased the splenic cell number as observed morphologically. Slightly increase in splenocyte density was also noted in the L-thyroxine treated bird where as melatonin treatment along with L-thyroxine increased more splenocytes density more in comparison to all experimental groups (Fig. 5).

## 4. Discussion

Inverse interrelationship and interdependency of pineal-thyroid function(s) was established in several tropical seasonally breeding birds (Haldar and Ghosh, 1988; Haldar and Rai, 1997; Maitra et al., 1996) and in a rodent, *F. pennanti* (Shavali and Haldar, 1998) in relation to reproduction. Pinealectomy induced hypothyroidism while melatonin injection to pinealectomized rodents restored it to control level (Shavali and Haldar, 1998). Further, interdependency of pineal-thyroid in relation with immune system of Indian tropical rodent, *F. pennanti* was also established by (Haldar and Singh, 2001). All these studies clearly put forward the importance of both the glands pineal and thyroid for not only reproduction, and perpetuation of species but also for immunity (Lewinski, 1990; Halder and Shavali, 1992).

It has been demonstrated that thymus growth is negatively influenced by removal of thyroid (Haldar and Singh, 2001). However, thyroid and its hormone $T_4$ are known to modulate the thymus dependent immune function (Fabris and Mocchegiani, 1985; Fabris et al., 1982; Ong et al., 1986) in mammals only.

Scanty literature on avian immune system led us to study for the first time the impact of melatonin and thyroid hormone (L-thyroxine) on immune functions of a tropical seasonally breeding avian species *P. asiatica*. Our data on the spleen weight showed that L-thyroxine treatment to the bird significantly increased the spleen weight, while,

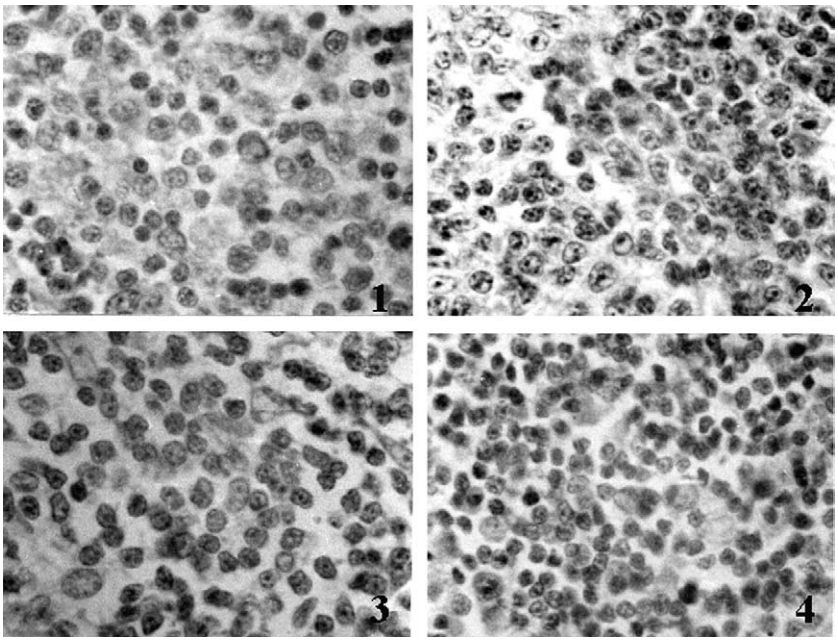


Fig. 5. (1) Histology of spleen of control bird showing splenic lymphocytes (L) during reproductively inactive phase (November to December) X 920. (2) Histology of spleen of melatonin treated bird, showing, increase of splenic lymphocytes (L) during reproductively inactive phase (November to December) X 920. (3) Histology of spleen of L-thyroxine treated bird, showing increase of splenic lymphocytes (L) during reproductively inactive phase (November to December) X 920. (4) Histology of spleen of L-thyroxine and melatonin treated bird showing more dense splenic lymphocytes (L) during reproductively inactive phase (November to December) X 920.

L-thyroxine treatment along with melatonin showed more additive stimulation. Scheift et al. (1997) and Dardenne et al. (1988) have reported that thyroid hormones modulate the endocrine function by causing epithelial cell proliferation of thymus (Fabris et al., 1989), which is known to synthesize thymic hormone (Gupta et al., 1983) in mammals but nothing is known for splenocyte proliferation/enlargement following L-thyroxine treatment to birds. Such a splenomegaly in our avian model following L-thyroxine and melatonin treatment is quite interesting. It led us to understand that splenomegaly in winter could be responsible for preventing birds from seasonal infections as winter is stressful for this birds (less food, shelter, and low temperature) when both the hormones are peripherally high.

Our data showed that L-thyroxine treatment either alone or with melatonin significantly increased the lymphocyte count as reported in rats and humans (Hassman et al., 1985). It has been suggested that thyroid hormone is more potent to enhance the immune function when present in vivo as it promotes maturation and differentiation of the lymphocytes (Fabris et al., 1995; Gala, 1991; Johnson et al., 1992).

Hence, we performed the lymphocyte proliferation assay of splenocytes cell of *P. asiatica* to the mitogen Con A. The ability of lymphocytes responds to mitogen by undergoing mitotic proliferation is a distinctive characteristics and this reflects the immune potential of the organism. Keast and Taylor (1982) showed that mammalian T-lymphocyte exposed to thyroid hormone increased their degree of activation as demonstrated by an increased response to phytoagglutinine (PHA).

Our observation on spleen histology presented that L-thyroxine treatment induced more cell proliferation than control bird while spleen histology of melatonin along with thyroxine treated birds showed more dense cellular architecture. Further, lymphoproliferative response of splenocytes to the mitogen Con A showed significant increase in blastogenic response of -thyroxine treated bird. However, melatonin treatment along with L-thyroxine significantly increased the splenocytes proliferative response when compared with lymphocyte proliferative response of birds treated only with melatonin.

In contrast to in vivo, supplementation of L-thyroxine in vitro significantly decreased the lymphoproliferative activity of splenocytes in vitro (Figs. 4A and B) while melatonin supplemented culture showed significantly increased lymphoproliferative response of splenocytes in vitro similar to in vivo (Set 1, Figs. 3A and B). Further, melatonin supplementation along with L-thyroxine brought back splenocyte proliferation to control level but was significantly less than noted in melatonin-supplemented group. This presents a differential effect of $T_4$ alone i.e. under in vivo condition (Exp. Set I) it has stimulatory effect, while under in vitro (Exp. Set II) it has inhibitory influence on splenocytes proliferation (Figs. 3A and B, Figs. 4A and B) demonstrated for the first time by us. It may also be suggested that such a stimulatory effect of $T_4$ on immunity in vivo could be an indirect one and might be via induction of some splenic hormone protein or via inhibiting other hormone/system as noted by Maestroni and Conti (1991) for thymus in rodents. Further, it could be that in the absence of normal internal physiological conditions of the birds, $T_4$

showed a negative effect on splenocytes proliferation in vitro. This is because some immune cells are capable of producing their own signal peptides thereby making in vitro results different from in vivo. However, melatonin maintained its lymphoproliferative effect under both the conditions.

The thyroid hormone is having receptors on lymphocytes and thymocytes in rats (Csaba et al., 1977) hence, a direct effect of thyroxine and melatonin, on lymphoid organ could be conceived. Further, melatonin receptors have also been detected on the circulating lymphocytes (Calvo et al., 1995; Liu and Pang, 1993; Pang and Pang, 1992; Poon and Pang, 1992) as well as on thymocytes and splenocytes (Lopez-Gonzalez et al., 1993; Martin-Cacao et al., 1993; Rafii-El-Idrissi et al., 1995) of mammals and humans. Hence, it could be proposed that there may be a common site of action through which melatonin and L-thyroxine synergies the thyroxine modulated immune function in vivo and vice versa. Such an intricate relation of thyroid and thymus function has been noted in tropical rodent (Haldar and Singh, 2001), which supports our finding in avian group for in vivo result.

Melatonin showed positive immunoenhancing properties both in vivo as well as in vitro, while thyroid hormone (L-thyroxine) showed immunoenhancing property in this bird either alone or along with melatonin under in vivo conditions only. In vitro thyroxine supplementation decreased the lymphoproliferative response of splenocytes, which suggested us to refer the action of L-thyroxine as differential. Therefore, immune cells exposed to various hormones under in vitro condition needs more attention to differentiate the direct versus indirect effect of various endocrine components on modulation of immune function.

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