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Oxidative Damage and Apoptosis Induction by L-thyroxine in the Spleen of a Tropical Bird *Perdicula asiatica:* Rescue by Melatonin

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Abstract

Avian thyroid gland is known to influence the immunity and reproduction in an opposite manner. In this study, we evaluated the immunostimulatory, anti-oxidative and anti-apoptotic roles of melatonin in a tropical bird, Indian jungle bush quail, *Perdicula asiatica*, having L-thyroxine (thyrotoxicity)-induced oxidative stress. Administration of L-thyroxine (100 µg/kg body weight) enhanced the thyroidal lipid peroxidase (LPO), with a parallel decrease in the levels of antioxidant enzyme (SOD, GPx, CAT, MDA & NO) activities. Cellular immune response (%SR) and humoral immune response (anti-KLH-IgG level) of splenocytes along with general hematological parameters (TLC, LC & HF/L ratio) decreased significantly upon L-thyroxine treatment. Further, decrease in circulatory anti-inflammatory cytokines IL-2 and TNFα suggested drastic effects of induced thyrotoxicity (elevated levels of T3 & T4) on immunity. Melatonin pre-treatment (25 µg/100g BW) during evening hours (prior to L-thyroxine treatment in the afternoon) for 30 days circumvented the deleterious effects of L-thyroxine-induced oxidative stress (level of Corticosterone) and apoptosis index of the avian spleen. Our results clearly indicate the potential of melatonin in rescuing/reducing the thyrotoxicity-induced oxidative damage to avian immunity.

Keywords: Anti-KLH IgG, Apoptosis, L-Thyroxin, Melatonin, Oxidative Stress

1. Introduction

Reports from mammals suggest a positive correlation between thyroxine and lymphoid organs as evident with marked increase in thymus size following thyroxine (T4) treatment[1]. In rodents, thyroidectomy resulted in hypoplasia of lymphoid organs[2] and L-thyroxine, being a catabolic hormone, induces oxidative load in tissues by increasing the free radical generation[3]. Thus, it can be speculated that in rodents L-thyroxine might be both beneficial as well as detrimental for the lymphoid organs. Reports regarding the effect of L-thyroxine on immunity of birds are lacking, though its effects on reproduction are well established[4]. Hence, in the present study we examined the oxidative stress on humoral and cell-mediated immune responses of the spleen following L-thyroxine treatment in a tropical avian species *Perdicula asiatica*, the Indian jungle bush quail. On the other hand, melatonin is a known antioxidant and also an immune-stimulator in birds[5,6]. Therefore, in the present study we examined the potential rescuing effects of melatonin on thyrotoxicity (by L-thyroxine) induced oxidative stress in general, and cellular (splenocyte percent Stimulation Rate - % SR) and humoral immunities (anti KLH IgG level) along with other damages (apoptotic index) caused to spleen in the tropical bird *Perdicula asiatica.*

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2. Materials and Methods

The experiments were conducted on adult male jungle bush quails, *P. asiatica* (body weight 40±5g) collected from the vicinity of Varanasi (Lat. 25°, 18′ N, Long. 83°, 01′ E). This bird is categorized under least endangered species and is a common game bird. The maintenance and reproductive seasonality of this bird have already been published in details[7]. This bird is a long-day breeder having Reproductively Active Phase (RAP) from April to July and a Reproductively Inactive Phase (RIP) from November to January[8]. The experiments were conducted during RIP. Wild-collected birds were acclimated to laboratory conditions of temperature and photoperiod for two weeks in an open-air aviary and then transferred to specially designed bird cages (25' x25' x30') to experience natural photoperiodic conditions as observed during the RIP (11 light; 13 dark and temp 13-21°C). The birds were fed with grains, millets and water *ad libitum*. Experiments were conducted in accordance with institutional practice and within the framework of Institutional Animal Ethics Committee under the purview of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the Government of India.

2.1 Experimental Design

Total of 40 adult male birds of similar age (as judged from full keratinization of beak) and body weight (40±5g) were randomly divided into four groups of 10 birds each. The group 1 served as control birds (10 nos.) and received daily subcutaneous (s.c.) injections of ethanolic saline (0.01% ethanol in Normal saline). Birds in group 2 received injections of L-thyroxine (i.p.) at a dose of 100 µg/kg body weight.[9] Birds in group 3 were given melatonin injections (25 µg/100 g body weight s.c.) 1 hour before lights were off[7]. Birds in group 4 had pre-injection of melatonin (previous day during evening hours) followed by L-thyroxine treatment the next morning. The duration of treatment was 30 consecutive days. From these groups estimation was performed in two sets. The first set of birds (n= 5 per sub-group) was used for the study of humoral immune response in terms of anti-KLH-IgG production and the second set (n= 5 per sub-group) was utilized for the study of cellular immunity function (%SR) and other immune parameters (TLC and LC, and HFL ratio), cytokines (IL2 and TNFα) assay as well as oxidative stress parameters (SOD, CAT, MDA, NO, and GPx of the spleen. The plasma was used for ELISA of hormones (corticosterone, T3, T4 and melatonin). The nutritional stress, if any, was checked by noting the initial and final body weights of the birds covering the entire course of the experiment, which was always not significant.

2.2 Drugs and Treatments

Melatonin, L-thyroxine, Concanavalin A (Con A) and keyhole limpet hemocyanin (KLH; antigen) were purchased from Sigma–Aldrich Chemicals, St. Louis, MO, USA. Melatonin was first dissolved in a few drops of ethanol and then diluted with normal saline up to the desired concentration for administration. L-thyroxine was dissolved in trace amounts of 0.01N NaOH and diluted with normal saline to make specific concentration. Melatonin was injected subcutaneously during the evening hours (between 17:30-18:00 h IST; approximately 1-1.5 h before lights were off), and L-thyroxine was injected intraperitoneally during the morning hours (11 am IST).

2.3 Sample Collection

Twenty-four hours after the last injection the birds were weighed and sacrificed during the night time under dim red light between 20:00 and 22:00 h, IST, under deep ether anesthesia. Blood was collected by decapitation in heparinized tubes and centrifuged at 3000 rpm for 20 min at 4°C. Plasma was separated and kept at–20 °C till hormone estimations were performed. Spleen was dissected out aseptically on ice, weighed and a part of the spleen was processed for the assay of blastogenic response of splenocytes while the remaining tissue was kept at –20°C for biochemical estimations.

2.4 Total Leukocyte Count,% Lymphocyte Count and Heterophil/Lymphocyte Ratio

Blood samples of the birds from all groups were collected in WBC pipettes, diluted 20 times in Turk's solution (2.0 mL glacial acetic acid, 0.1 g mercuric chloride, one drop aniline, and 0.2 g Gentian violet) to count the total number of leukocytes (no/mm^3) using the Neubauer's chamber /(haemocytometer (Spencer, USA) under Nikon (Japan) binocular microscope[7].

A thin film of blood was drawn on a slide, stained with Leishman's stain for differential leukocyte

(lymphocyte) count under oil immersion lens of Nikon E200 microscope (Japan). Percent lymphocyte count (no./mm^3) was determined from the total and differential leukocyte count by using the following formula:

$$\% \text{ Lymphocyte Count} = \frac{\text{TLC x Lymphocyte percentage}}{100}$$

The heterophil/lymphocyte (H/L) ratio was obtained from blood smear following the method of Ots *et al.*[10] using a Nikon E200 microscope.

2.5 Analysis of Oxidative Stress Parameters

2.5.1 Lipid Peroxidation (LPO) Assay

Spleens were dissected out, weighed and homogenized in 20 mM Tris HCl buffer (pH 7.4). 10% homogenates were centrifuged for 15 min at 3000 × g at 4°C. The supernatant was subjected to Thiobar-Bituric Acid (TBA) assay by mixing with 8.1% sodium dodecyl sulfate, 20% acetic acid, 0.8% TBA and boiling for 1 h at 95°C[11,12]. The reaction mixture was immediately cooled and vigorously shaken with n-butanol and pyridine reagent (15:1), then centrifuged for 10 min at 1500 × g. The absorbance of the upper phase was measured at 534 nm in a Spectrophotometer (Bio-Tek, USA). Total Thiobar-Bituric-Acid-Reactive Substances (TBARS) were expressed as nMol /g tissue, taking 1, 1, 3, 3-Tetra-Ethoxy Propane (TEP) as standard. The standard curve was calibrated using a 10 nM concentration of TEP.

2.5.2 Colorimetric Determination of Superoxide Dismutase (SOD) Activity

Homogenates of spleen were centrifuged for 30 min at 12,000×g at 4°C (Heraus, Germany). The supernatant was separated and again centrifuged for 60 min at 12,000 × g at 4°C, then processed for enzyme activity using nitrite formation by superoxide radicals[13-15]. The homogenate,0.1 mL, was added to 1.4 mL of reaction mixture comprising 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride, and 50 mM Ethylene Diamine Tetraacetic Acid (EDTA), followed by a brief pre-incubation at 37°C for 5 min in CO_2 incubator (HeraCell, Germany). Riboflavin, 0.8mL, was added to all samples (with a control containing buffer instead of sample) and then exposed to two 20W fluorescent lamps fitted parallel to each other in a specially designed wooden box coated with aluminum foil. After 10 min of exposure, 1 mL Griess reagent was added and absorbance of the color generated was measured at 543 nm. One unit of enzyme activity is defined as the amount of SOD required to inhibit 50% of nitrite formation under assay conditions.

2.5.3 Determination of Catalase (CAT) Activity

Catalase activity was estimated by the method of Aebi[16] by mixing 500 µL of the sample with 10 µLof absolute ethanol, mixed thoroughly in a motor-driven vortex apparatus and kept on ice for 30 min. Triton X-100, 50 µL, was gently mixed with 450 µL of the sample prepared in absolute ethanol. The sample, 100 µL (Ethanol + sample + Triton X-100) was mixed with 2.8 mL of 50 mM phosphate buffer (pH 7.0). The change in absorbance was measured at 240 nm promptly after adding 100 µL of 60 mM H_2O_2. The enzyme activity was expressed in pKat/mg protein.

2.5.4 Total Nitrite and Nitrate Concentration for Nitric Oxide (NO) Determination

Total nitrite and nitrate concentration, an indication of NO synthesis, was measured in spleen following the method of Sastry *et al*[17]. Tissue homogenate at 5% was prepared in 0.01 M phosphate buffer, pH 7.4. To 100 µL of each sample 400 µL of carbonate buffer was added followed by a small amount (0.15g) of activated copper – cadmium alloy powder and incubated at room temperature with thorough shaking. The reaction was stopped by the addition of 100 µL of 0.35 M NaOH followed by 120 mM $ZnSO_4$ solution under vortex and allowed to stand for 10 min. Tubes were then centrifuged at 8000 rpm for 10 min. The clear supernatant, 100 µL, was transferred to a microplate (in quadruplicate) and Griess reagent was added. After 10 min, the absorbance was read at 545 nm in an ELISA plate reader (ELx-800, Biotek Instruments, and Winooski VT, USA). A standard graph was plotted against different concentrations (0, 20, 40, 60, 80, and 100 µM) of KNO_3.

2.5.5 Glutathione Peroxidase Activity Assay

Protein from spleen homogenates, 150 µg each, were mixed in a reaction mixture comprising 50 mM/L PBS (pH 7), 1 mM/L EDTA, 1 mM/L sodium azide, 0.5 mM/L NADPH, 0.2 mM/L reduced glutathione and one unit of glutathione reductase. The reaction was allowed

to equilibrate for 1 min at room temperature. H_2O_2, 0.1 mM/L, was added to initiate the reaction and the decrease in the OD was recorded at 340 nm for 3 min at 30 s intervals. The GPx activity was calculated as nM/L NADPH oxidized/min/mg protein according to Mantha, *et al*[18].

2.6 Blastogenic Response of Splenocytes (% Stimulation Ratio)

Spleens were minced in ice-cold PBS inside a laminar hood and erythrocytes were lyzed by hypotonic shock using equal volumes of cold ammonium chloride Tris buffer (0.5% Tris hydroxymethylene aminomethane, BDH, UK) and 0.84% NH_4Cl, mixed in 1:10 ratio; pH 7.2. Viability and cell count were determined by trypan blue dye exclusion method and Turk solution, respectively. Splenocyte suspensions were seeded in 35 mm sterile culture petri plates at a density of $2x10^6$ cells per mL in complete RPMI 1640 medium containing penicillin 100 U/mL, streptomycin 100 mg/mL and 10% fetal calf serum (Sigma, USA). The cell suspension was divided into one mL each (having $2x10^6$ cells/mL) per plate with and without the T-cell mitogen Concanavalin A (Con A) at 10 mg/mL. The plates were kept in 37°C incubator with 5% CO_2 atmosphere for 48 hr.

2.7 Cell Harvesting

Dimethyl thiazolyl diphenyl tetrazolium salt MTT (5mg/mL) 100 µL was added to each culture 3 hr before the scheduled harvesting at 48 h and the OD was read at 590 nm.

$$\%\,SR = \frac{\text{OD of Mitogen} - \text{stimulated cells}}{\text{OD of Basal cells}} \times 100$$

2.8 Anti-KLH-IgG Estimation for Humoral Immune Status

The anti-KLH-IgG estimation assay was performed following the method of Dames *et al.*[19] with some modifications which has been published elsewhere[20]. Since we performed this KLH immunized humoral assay for the first time in birds, a separate experiment with birds (n=10) was planned and a single subcutaneous injection of 150 µg of KLH (Sigma–Aldrich) in 0.1 mL sterile normal saline was administered for immunization and noted as day 0. The birds were sacrificed under deep anesthesia and trunk blood was collected for assay of anti-KLH IgG on the day 5 and 10 (post-immunization), in order to standardize the optimal result of the assay. These periods were chosen to capture basal (5 days) and peak (10 days) levels of anti-KLH IgG. Then 24 hr after the last injection given to the second experimental set of the birds, a single subcutaneous injection of 150 µg of KLH (Sigma–Aldrich) in 0.1 mL of sterile normal saline was administered for immunization and noted as day 0. The trunk blood was allowed to clot and after 1 hr, the clots were removed and samples were centrifuged (at 4°C) for 30 min at 700×g. Serum was separated and aliquots were stored at −20°C until assayed for anti-KLH-IgG.

2.9 ELISA for Anti-KLH-IgG

The humoral immune status was assessed by measuring plasma anti-KLH-IgG concentrations using ELISA[20]. In brief, the microtiter plates were coated with KLH antigen (0.5 mg/mL in sodium bicarbonate buffer) by overnight incubation at 4°C. The next day, plates were washed with Phosphate Buffered Saline containing 0.05% Tween 20 (PBST), blocked with 0.5% non-fat dry milk in PBST overnight at 4°C and washed again with PBST. The serum samples were diluted 1:50, 1:100, 1:200, 1:400 and 1:800 in PBST, and finally 150 µL of each serum dilution was loaded in duplicate in antigen-coated microtiter plate. Similarly, the positive control (birds those have prior been exposed to KLH showing robust immune reaction) and negative control samples (birds never exposed to KLH) were diluted and loaded in wells in duplicate. The plates were sealed and incubated at 37°C for 3 h followed by PBST wash. Secondary antibody (alkaline phosphatase conjugated, anti-mouse IgG; SC-2320, Santa Cruz Biotek, USA) was added to each well in a dilution of 1:500; and plates were incubated again at 37°C for 1 hr followed by washing with PBST. p-nitrophenyl phosphate (enzyme substrate) at 1 mg/mL in diethanolamine buffer (Sigma–Aldrich),150 µL, was added to each well and the plates were kept in dark, the enzyme-substrate reaction was terminated by adding 50 µL of 1.5 M NaOH to each well. The Optical Density (OD) of each well was determined using a microplate reader equipped with a 405 nm filter (ELx-800, Biotek Instruments, Winooski, VT, USA) and the average OD was calculated for each sample in duplicate. The mean for each sample was calculated and expressed as a percentage of the positive control mean (% plate positive).

2.10 ELISA of Hormones and Cytokines

2.10.1 Plasma T3 and T4

The ELISA of T3 and T4 was performed according to the manufacturer's instruction given on the kit (Diametra: DKO045). Signals were analyzed at 450 nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation). The sensitivity of the T3 and T4 assays were 5 ng/mL and 0.4 µg/mL, respectively. Inter- and intra-assay variations for the T3 assays were <9.1% and <10.7%, respectively. Similarly, the inter- and intra-assay variations for the T4 assay were<8.42% and <8.16%, respectively. All samples were assayed in a single lot, and samples, standards, and replicates were assayed in duplicate.

2.10.2 Plasma Corticosterone ELISA

Corticosterone was measured using an ELISA kit (kind gift from Prof. T. G. Srivastava, National Institute for Health and Family Welfare, New Delhi, India) according to the manufacturer's instructions. Plasma samples were thawed, and pipetted out into a microplate. Following incubation with different reagents provided in the kit, finally the plates were read on a microplate reader at 405 nm, and values were determined by extrapolation from a standard curve. The recovery, accuracy, and sensitivity of the corticosterone assay were 95%, 99.1%, and 0.27 g/dL, respectively. Inter- and intra-assay variations were 3.38–5.56% and 5.69–7.84%, respectively. All samples were assayed in a single lot and samples, standards, and replicates were assayed in duplicate.

2.10.3 Plasma Melatonin

Melatonin ELISA was performed according to the manufacturer's protocol provided in the kit (Uscn Life Science Inc., Wuhan, Hubei, China). The kit had sensitivity of 4.68 pg/ mL, as per the manufacturer's indtructions. The intra- and inter-assay variations were <10% and <12%, respectively. Recovery percentage was between 90 and 115%.

2.10.4 Plasma Interleukin-2 (IL-2)

Sandwich ELISA was performed to quantify plasma level of IL- 2 in all the four groups according to manufacturer's instruction (Immunotech, France). Intra-assay variation was between 3.3 and 7.2% and inter-assay variation was between 6.2 and 8.2%; sensitivity; 5 pg/mL and recovery were between 80 and 132%.

2.10.5 Plasma TNFα

Sandwich ELISA was performed to quantify plasma level of TNFα in all the four groups according to manufacturer's instruction (Immunotech, France). Intra-assay variation was between 4.5 and 6.2% and inter-assay variation was between 4.3 and 5.6%; sensitivity 5 pg/mL and recovery was between 60 and 150%.

2.11 Apoptotic Index of Splenocytes

The apoptotic cells were analyzed microscopically following Acridine Orange and Ethidium Bromide (AO-EtBr) double staining method as described elsewhere[21]. AO-EtBr dye of volume 0.01 mL (1×) was mixed gently with 0.2 mL of the diluted sample (1× 10^6 cells/mL in PBS). A drop of this mixture was placed underneath cover-slip on a clean slide and cells were observed immediately in a fluorescent microscope (Leitz MPV3, Wetzlar, Hesse, Germany). A minimum of 200 cells was counted in every sample to observe cell death.

$$\frac{\text{No. of apoptotic cells (early + late apoptotic)}}{\text{Total no. of cells counted}} \times 100$$

2.12 Statistical Analysis

All statistical analyses were performed using SPSS version 17.0. The data were analyzed by performing one-way ANOVA followed by multiple comparisons by Tukey's HSD (Honest Significant Difference) multiple range tests. The values are expressed as mean ± SEM. A p value of <0.05 was considered statistically significant. Correlation analysis was done taking into account the correlation coefficient = r (which denotes the strength of the relationship) between the H/L ratio, serum anti-KLH IgG and serum corticosterone level. The correlation analysis was also done for serum melatonin and corticosterone as well as serum TNF-α and IL-2 levels. Microsoft Excel program was used for the correlation analysis.

3. Results

3.1 Relative Weight of Spleen

Melatonin-treated birds showed a significant ($p<0.01$) increase in spleen weight when compared to control.

Further, birds receiving melatonin pre-treatment followed by thyroxine showed no significant difference in spleen weight when compared to L-thyroxine only treated birds (Figure 1).

3.2 PlasmaT4 and T3 Levels

Melatonin treatment significantly ($p< 0.01$) decreased T3 (Figure 2a) and T4 levels (Figure 2b). Significant increase in plasma T3 and T4 levels of L-thyroxine treated birds were found. Melatonin pre-treatment followed by thyroxine decreased the plasma T3 and T4 levels significantly ($p< 0.05$) when compared with L-thyroxine only treated birds.

3.3 Plasma Melatonin and Corticosterone Levels

Plasma melatonin level increased ($p<0.01$) while corticosterone level decreased significantly ($p<0.01$) in the melatonin-treated birds (Figure 3a and 3b) when compared to controls. L-thyroxine treatment significantly lowered the plasma melatonin ($p<0.01$) and elevated the corticosterone levels ($p<0.01$). Melatonin pre-treatment followed by thyroxine restored the plasma melatonin level and decreased the corticosterone level ($p<0.05$) when compared to L-thyroxine only treated birds.

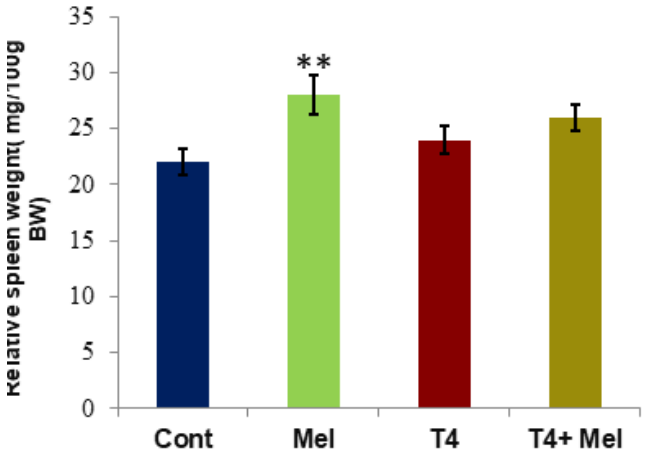

Figure 1. Effect of melatonin pre-treatment and L-thyroxin on weight of spleen during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel.

3.4 Plasma IL-2 and TNFα Levels

Significant ($p<0.01$) decrease in plasma levels of IL-2 and TNFα were found in L-thyroxine treated birds, while it was significantly ($p<0.01$) increased in melatonin treated

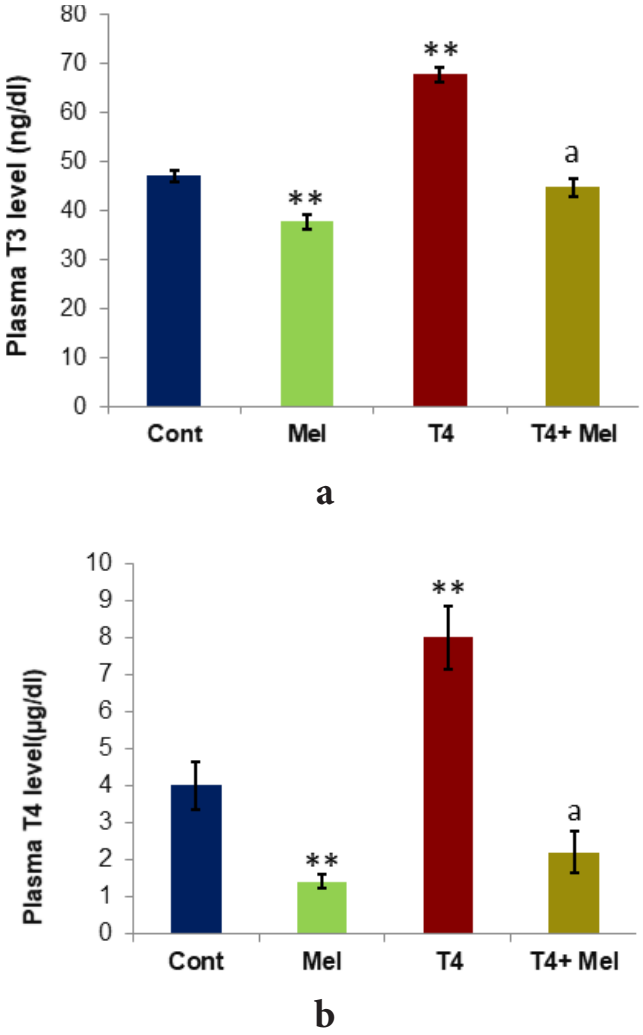

Figure 2 (a). Effect of melatonin pre-treatment and L-thyroxine on plasma T3 level during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean+SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(b).** Effect of melatonin pre-treatment and L-thyroxine on plasma T4 level during reproductively inactive phase (Nov-Jan) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel.

birds. Melatonin pre-treatment followed by thyroxine significantly ($p<0.05$) enhanced the plasma IL-2 and TNFα levels respectively when compared with L-thyroxine only treated group (Figure 3c and 3d).

3.5 Blastogenic Response of Splenocytes (% SR)

Both melatonin and L-thyroxine treated birds had a significantly ($p<0.05$ and $p<0.01$ respectively) increased blastogenic response of splenocytes in terms of % SR. Further, melatonin pre-treatment followed by thyroxine treated birds also showed a significant ($p<0.05$) increase in the % SR of splenocytes when compared with L-thyroxine only treated group (Figure 3e).

3.6 Anti-KLH-IgG Estimation for Humoral Immune Status

Anti-KLH-IgG titer was found to be significantly ($p<0.01$) increased at 10 days compared to 5 days. Hence, in all experimental groups results of 10 days were recorded. Melatonin treatment significantly ($p<0.01$) increased the serum anti-KLH-IgG. L-thyroxine administration significantly decreased ($p<0.01$) the anti-KLH IgG. Melatonin pre-treatment followed by L-thyroxine recovered the anti-KLH-IgG titer ($p<0.05$) when compared with the L-thyroxine only treated group (Figure 3f).

3.7 Oxidative Stress Parameters

3.7.1 TBARS Levels and Activity of Superoxide Dismutase (SOD)

Melatonin treatment caused a significant ($p<0.01$) decrease in TBARS level while the SOD activity in spleen was elevated (Figure 4a and 4b). L-thyroxine treatment caused a significant increase in TBARS and suppressed the activity of SOD in spleen of birds when compared to controls. Melatonin pre-treatment followed by L-thyroxine restored the TBARS ($p<0.05$) and SOD activity when compared to L-thyroxine treated birds.

3.7.2 Catalase Activity (CAT)

Melatonin treatment significantly ($p<0.01$) increased the CAT activity in spleen (Figure 4c) while a significant decrease ($p<0.01$) was noted in L-thyroxine treated birds when compared with control birds. Melatonin pre-treatment followed by L-thyroxine restored the CAT activity ($p< 0.05$) when compared to L-thyroxine treated group.

3.7.3 Glutathione Peroxidase Activity (GPx)

Melatonin treatment caused a significant ($p<0.001$) increase in GPx activity in spleen, while a significant decrease in GPx activity ($p<0.01$) was noted in L-thyroxine treated birds when compared with control birds (Figure 4d). Melatonin pre-treatment followed by thyroxine significantly ($p<0.05$) increased the GPx activity when compared to L-thyroxine treated group.

3.7.4 Nitrite and Nitrate Ion Estimation

Melatonin treatment caused a significant ($p<0.05$) decrease in nitric oxide (NO) level in spleen (Figure 4e) while L-thyroxine caused a significant increase ($p<0.001$) in NO level when compared with control birds. Melatonin pre-treatment followed by L-thyroxine significantly ($p<0.05$) increased the NO level when compared to thyroxine only treated birds.

3.8 Hematological Parameters (TLC, %LC and H/L ratio)

Treatment of L-thyroxine had no effect on TLC (Figure 5a) but increased % LC (Figure 5b) and H/L ratio (Figure 5c) significantly. Melatonin treatment increased the TLC, % LC ($p<0.01$) and lowered the H/L ratio. Birds with pre-treatment of melatonin followed by L-thyroxine showed significantly higher values for TLC, % LC when compared with L-thyroxine only treated birds. The H/ L ratio in the melatonin pre-treated group were similar to control birds.

3.9 AO-EtBr Staining of Splenocytes and Apoptotic Index

A significant ($p<0.01$) increase in apoptotic index (%) of splenocytes was noted in L-thyroxine treated group when compared with control (Figure 6). Melatonin treatment produced no significant difference in apoptotic index. However, melatonin pre-treatment followed by L-thyroxine significantly ($p<0.05$) decreased the apoptotic index when compared with L-thyroxine only treated group.

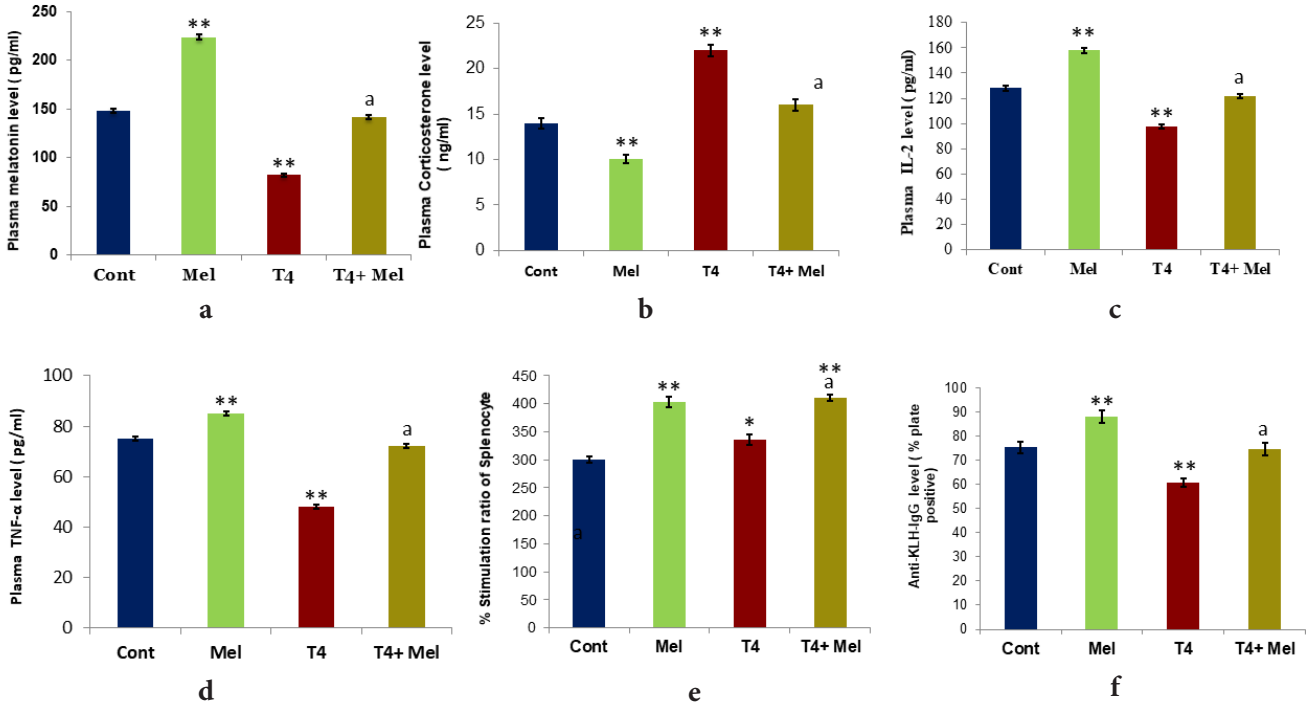

Figure 3 **(a).** Effect of melatonin pre-treatment and L-thyroxine on plasma melatonin during reproductively inactive phase (Nov-Jan) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(b).** Effect of melatonin pre-treatment and L-thyroxine on plasma corticosterone during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(c).** Effect of melatonin pre-treatment and L-thyroxine on plasma interleukin 2 (IL-2) level during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(d).** Effect of melatonin pre-treatment and L-thyroxine on tumor necrosis factor alpha (TNF α) level during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(e).** Effect of melatonin pre-treatment and L-thyroxine on blastogenic response of splenocytes challenged with T cell mitogen Concanavalin A (Con A) represented as % stimulation ratio (%SR) during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{*}p < 0.05$ indicatesCont Vs T4, $^{**}p < 0.01$ indicates Cont Vs Mel and T4+Mel, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(f).** Effect of melatonin pre-treatment and L-thyroxine on anti-KLH IgG levels in plasma during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel.

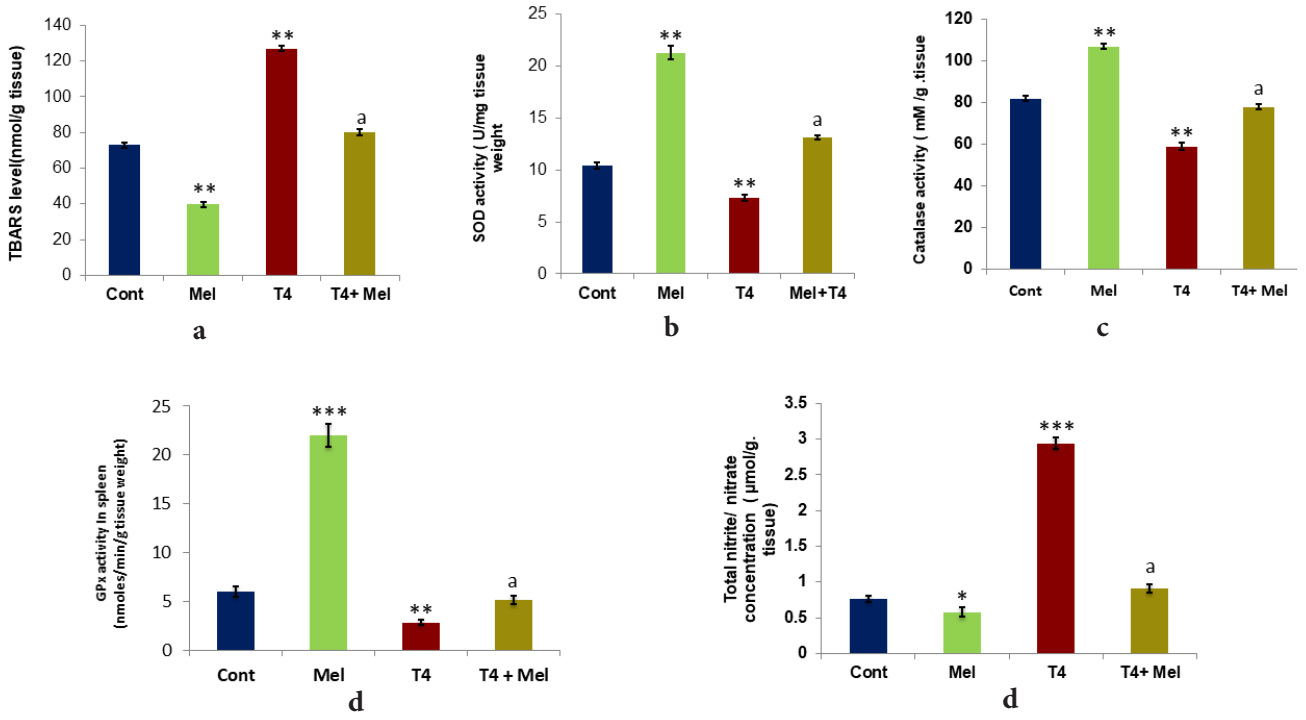

Figure 4 **(a).** Effect of melatonin pre-treatment and L-thyroxine on lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS) during reproductively inactive phase (Nov-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(b).** Effect of melatonin pre-treatment and L-thyroxine on and activity of superoxide dismutase (SOD) during reproductively inactive phase (Nov-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(c).** Effect of melatonin pre-treatment and L-thyroxine on activity of catalase (CAT) expressed as pKat/mg protein during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p< 0.01$ indicates T4 Vs. T4+Mel. **(d).** Effect of melatonin pre-treatment and L-thyroxine on activity of Glutathione peroxidise (GPx) during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs.T4, $^{***}p < 0.001$ indicates Cont Vs.Mel, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(e).** Effect of melatonin pre-treatment and L-thyroxine on total nitrite/nitrate concentration in tissue homogenates during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{*}p < 0.05$ indicates Cont Vs. Mel, $^{***}p < 0.001$ indicatesCont Vs.T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel.

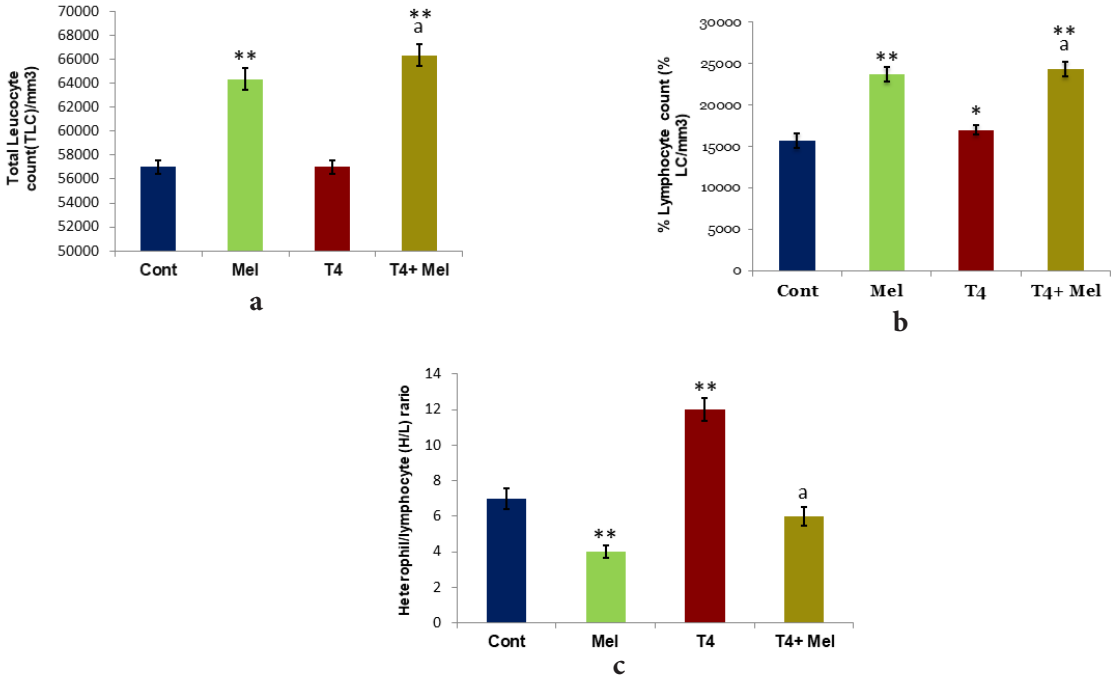

Figure 5 **(a).** Effect of melatonin pre-treatment and L-thyroxine on total leukocyte count (TLC) during reproductively inactive phase (Nov.Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4+Mel, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(b).** Effect of melatonin pre-treatment and L-thyroxine on % lymphocyte count (% LC) during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{*}p < 0.05$ indicates Cont Vs. T4, $^{**}p < 0.01$ indicates Cont Vs. Mel and T4+Mel, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel. **(c).** Effect of melatonin pre-treatment and L-thyroxine on heterophil/lymphocyte ratio (H/L ratio) during reproductively inactive phase (Nov-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. Mel and T4, 'a' $p< 0.01$ indicates T4 Vs. T4+Mel.

3.10 Correlation Analysis

H/L ratio showed a positive correlation (Figure 7a) with plasma corticosterone (r = 0.88, $p<0.001$) while plasma corticosterone depicted a negative correlation (Figure 7b) with anti-KLH IgG (r = -0.940, $p<0.01$). Plasma melatonin correlated negatively (Figure 7c) with plasma corticosterone (r = -0.940, $p<0.001$). Plasma IL-2 and TNFα (Figure 7d) showed a significant positive correlation (r = 0.913, $p<0.001$).

4. Discussion

Thyroid hormones have been reported to play a major role in the regulation of important physiological processes such as sexual maturation[22] breeding cycle[23] metabolism and migration[24] in birds. Seasonality plays a major role in the physiology of most of the birds as certain hormones present seasonal variation especially the reproductive hormones. Being no exception thyroid hormone also exhibits variations during summer and winter months hence strongly correlated with reproduction of birds[25]. The thyroid hormones have been reported to influence immune status of seasonally breeding rodents and lung associated immune functions of birds. Partial studies on *P. asiatica* showed apositive effect of L-thyroxine and melatonin on the proliferative potential of splenocytes[7]. On the other hand melatonin, being an established immune-stimulatory hormone, increased the % SR of avian splenocytes[7].

We found a similar response of melatonin and L-thyroxine treatment alone in regard to immune parameters, and free radical loads. But we more strongly

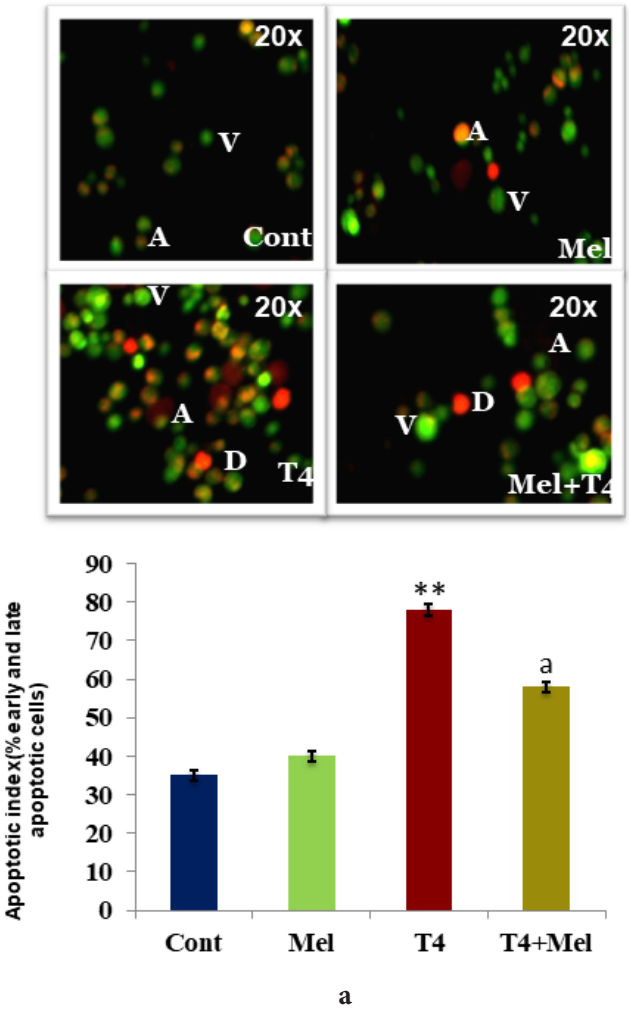

Figure 6. Acridine orange–Ethidium bromide (AO–EtBr) double staining of splenocytes showing apoptosis. Apoptotic cells (orange) are denoted by the letter "A" while the green are normal viable cells denoted by "V". The red depicts dead cells denoted by the letter "D". Effect of melatonin pre-treatment and L-thyroxine on apoptotic index of splenocytes during reproductively inactive phase (Nov.-Jan.) of jungle bush quail *Perdicula asiatica*. Values represent Mean + SEM, n = 5. Cont- Control, Mel- Melatonin, T4- L-Thyroxine, Mel+T4- Melatonin pre-treatment plus L-Thyroxine. $^{**}p < 0.01$ indicates Cont Vs. T4, 'a' $p < 0.01$ indicates T4 Vs. T4+Mel.

presented the support on the above statement based on the circulatory level of cytokines and hormones, and apoptotic index of the splenocytes. Interestingly, combination of melatonin pretreatment and L-thyroxine treatment showed induction of % SR and % lymphocyte count. Humoral immunity response in term of Anti-KLH IgG generation indicates the production of immunoglobulins upon immunogenic challenge by keyhole limpet hemocyanin (KLH) which is a proteinaceous antigen produced by sea snails. We, therefore, for the first time report the anti-KLH IgG titer as an important marker of humoral immune response in birds in general and in a tropical bird *P. asiatica* especially. The increased anti-KLH IgG titers following melatonin and L-thyroxine treatment suggested a positive effect of those hormones towards immune function. Looking into this aspect of L-thyroxine, and correlating it with immune function, we could suggest a positive feedback between thyroxine and general immune status.

Thyroxine, being a catabolic hormone, increases the basal metabolic rate and thereby increases O_2 consumption. L-thyroxine induces oxidative stress in many tissues like testes, kidney and even brain[9,26,27]. The increase in lipid peroxidation following L-thyroxine treatment clearly indicates an accumulation of free radicals and subsequent damage to the membrane lipids. The elevated level of corticosterone in birds is another indicator of stress which further strengthens the fact that L-thyroxine triggers a state of oxidative stress in the spleen. Melatonin, a strong antioxidant, alone treatment always had a positive impact on immunity. It reduced oxidative stress and thereby apoptosis at tissue level. It also decreased circulatory corticosterone, hence reduced stress. Melatonin decreased the plasma corticosterone levels thereby alleviating the stressed condition in the spleen and relieved the tissue from disrupted redox homeostasis.

Therefore, a synergistic effect of melatonin and L-thyroxine on spleen functions, thereby on immunity, was noted. It, on one hand, may have some oxidative stress generated by L-thyroxine treatment that may pose a threat to the redox homeostasis of spleen that was taken care by melatonin treatment. Oxidative stress and accumulated free radicals in the spleen of birds further activates downstream signaling molecules.

Cytokines serve as a mediator of physiological responses like inflammation. Circulatory interleukin 2 (IL-2) and Tumor Necrosis Factor Alpha (TNFα) are anti-

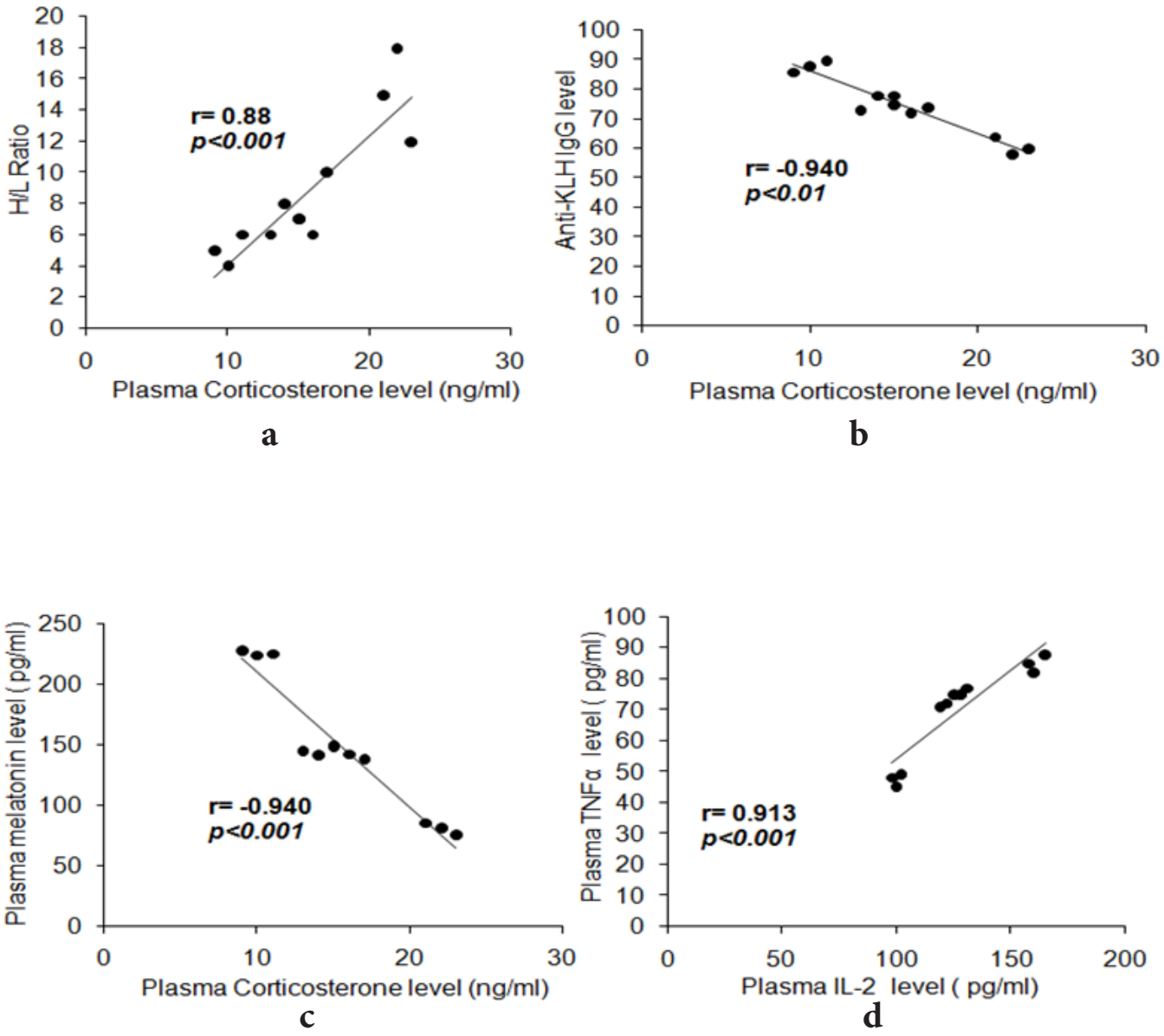

Figure 7. Correlation analysis between **(a)** H/L ratio and plasma corticosterone, **(b)** anti-KLH IgG levels and plasma corticosterone, **(c)** plasma melatonin and plasma corticosterone and **(d)** plasma TNF α and IL-2 being presented. Points are denoted with individual H/L ratio, anti-KLH IgG levels (% plate positive), corticosterone (ng/mL), melatonin (pg/mL), TNF α (pg/mL) and IL-2 (pg/mL) concentrations. The differences were considered significant when $p<0.05$. Positive and negative correlations were depicted on values of correlation coefficient = r, which measured the strength of the relationship.

inflammatory cytokines which showed synergism with melatonin, suggesting a cell-protective effect. L-thyroxine treatment suppressed both the anti-inflammatory cytokines indicating a shift of the cells towards an inflammatory condition. This is because oxidative stress invokes inflammation and the inflammatory responses that further help in generation of free radicals in the cells.

Apoptosis, of the physiological cell death, is often triggered by oxidative load. In our study the AO-EtBr staining depicted an increase in apoptotic cells following L-thyroxine treatment which indicates the cellular damage and decrease of splenic functions. Melatonin being an established anti-apoptotic agent[21] proved beneficial in reducing the apoptosis in the spleen of birds under thyrotoxicity-induced oxidative stress. Melatonin

treatment restored the proper functionality and viability of splenocytes thereby improving the cell and humoral-immunity in quails as well.

It can be summarized that L-thyroxine, besides its various effects on reproduction, induces thyrotoxicity in birds which influences splenic function and thus humoral- and cell-mediated immunity. At optimal level of thyroxine[7] is necessary for the proper immune and reproductive functions, yet it can induce oxidative load when administeredcor may be under natural condition (due to pesticides/insecticides used in fields as this bird is a graminivorous) that could be deleterious for many physiological functions of the birds including immunity.

Thyrotoxicity is now a common global phenomenon due to water pollution and indiscriminate use of pesticides in agriculture. Increased plasma thyroxine might be affecting the spleen functions by augmenting the oxidative load. Melatonin being an endogenous hormone of the body regulates a wide plethora of functions. The anti-apoptotic and anti-oxidative actions of melatonin in thyrotoxic conditions of birds definitely proves to be beneficial in reducing the generated oxidative load and immune deficiency. Therefore, melatonin may have a future therapeutic application for treatment of hyperthyroidism or induced thyrotoxicity in order to protect the organs of high physiological importance like gonads, spleen and liver.

5. Conclusion

Thyroid gland and its hormone thyroxine are important for the proper functioning of lymphoid organs. Thyroxine, being calorigenic in nature, increases oxygen uptake and thereby subsequent generation of free radicals which damages spleen cell and results in reduction of cell and humoral immunity along with induction of splenic apoptosis. This sort of effect is being reported for the first time in a tropical bird. This adverse effect can be minimized by melatonin treatment. Being an active antioxidant and free radical scavenger melatonin suppresses the generated oxidative load and ameliorated the free radical-induced damage and apoptosis in the spleen. Hence, it can be speculated that a treatment of melatonin which has no known side effects might be effective in neutralizing the negative aspect of thyroxine while its positive role towards the lymphoid organs remains unaltered.

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The authors report no conflict of interest either financial or personal.

7. Contributions

Vijay Kumar Verma carried out the experiments, and data analysis. Prof. C. Haldar was the supervisor and Ms. S. Pal helped in preparation of the manuscript.

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