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Research Article

Seasonal Cytokine Production and Combinatorial Effect of Recombinant Cytokines and Melatonin on Peripheral Blood Mononuclear Cells Proliferation

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Abstract

The immune system is influenced by various internal (like hormones) and external (environmental: seasons) factors in which melatonin plays a vital role. Melatonin modulates seasonal immunity by influencing the cell-mediated immune function like cytokine secretion and cellular proliferation in rodents, however, there is a lack of information that how melatonin modulates the secretion of different cytokines in different seasons and directs the cellular proliferation in presence of specific cytokine. Therefore, in the present study, we elucidated the effect of seasonal melatonin variation on peripheral cytokines (IL-2, IL-6, and TNF-α) level and proliferative response (%SR) of peripheral blood mononuclear cells (PBMCs). The effect of recombinant cytokines (rIL-2, rIL-6, and rTNF-α) on the proliferative response of PBMCs was also noted in presence of melatonin under in vitro condition. As a result, we observed that high endogenous melatonin causes an increased level of IL-2, and IL-6 and down-regulates TNF-α during the winter season. Melatonin significantly modulates the proliferative response of PBMCs in presence of rIL-2, rIL-6 and rTNF-α by enhancing the immunomodulatory effect of rIL-2 and rIL-6 and by diminishing immunosuppressive effect of rTNF-α in goat. In conclusion, we may propose that melatonin maintains immune-homeostasis by modulating circulatory cytokines levels during different seasons. Melatonin might influence the secretion of immunomodulatory cytokine (i.e. IL-2 and IL-6) and also regulate the immuno-inhibitory action of TNF-α to modulate cell-mediated immune function.

Keywords: Season; Melatonin; Cytokines; Recombinant Cytokines; Ruminant; Immune Function

Introduction

The Seasonal variation of melatonin levels has been suggested due to photoperiodic variations [1]. Photoperiodically influenced melatonin during different season modulates activities of the physiological system (reproduction and immunity) in the seasonally breeding animals [2,3]. The seasonal variation of immunological functions reflects the modulatory potential of melatonin on the immune system especially T-cell function and cytokine levels [4,5]. The extreme environmental factors confront the animals which cause stress [6] and immunocompromised states and ultimately lead to a higher mortality rate, especially in small ruminants. Melatonin regulates immune responses by influencing different cytokines [5]. Cytokines are known to possess functional attributes like pleiotropism, synergisms, redundancy and antagonism with the other cytokines and they act as a communicating agent between immune cells [7]. In domestic animals like goats, multiple studies have been carried out in the regulation of seasonal reproductive activity by melatonin [1]. Kaushalendra and Haldar have first suggested a positive correlation between melatonin and immunological responses in Indian goat [8]. Age-related declined in the immune responses has also been suggested in goat. It is also due to a decline melatonin level and increased nitrosative stress in lymphatic organ spleen while aging [9].

However, there is a lack of report which could give an insight into seasonal melatonin variation on circulatory cytokine level and their effect on proliferative responses of immunocompetent cells (PBMCs). Therefore, in the present study, we attempted to explore the effect of seasonal melatonin levels on the circulatory level of cytokines (IL-2, IL-6 and TNF-α) in plasma during summer and winter seasons. The effect of cytokines on the proliferative response of PBMCs was checked by using recombinant cytokines (rIL-2, rIL-6 and rTNF-α) in *the in-vitro* condition alone and the presence of melatonin.

Materials and Methods

All the experiments were performed following institutional practice and within the framework of revised animals Act of 2007 of Govt. of India on animal welfare (Committee for Control and Supervision of Experiments on Animals; CPCSEA).

Animal maintenance

The experiments were conducted with 7 adult goats (Age approximately 3-4 years, weight 25 ± 2 kg) during summer (June) and winter (December) month of Varanasi, (latitude 25° 18'N and longitude 83° 1'E). Animals were kept in the animal house and were provided with an *ad libitum* supply of food (hay, protein grains, mineral cake, and seasonally available green leaves and grasses) and water. The health of the goats was monitored by routine check-up of body temperature (normal rectal temperature: 102.5 - 103°F) and rumen movement by an authorized veterinary doctor. Prophylactic measures were adopted in terms of vaccination against enterotoxaemia, foot and mouth diseases and *peste des petits* ruminant (PPR). The goats were treated with anthelmintics twice per year and 0.5% solution of malathion (acaricidal baths) was sprayed externally at the interval of 2 months as described by Chowdhury., *et al* [10]. No seasonality in any type of infection was observed during the study period.

Blood sampling

Blood was collected from the jugular vein of animals within 15 - 20 minutes applying minimal stress and during night-time under dim red light (less than 1 lux at a distance of 20 cm) to avoid direct illumination to the eyes of goats throughout the experiment. Blood was collected in heparinized (anticoagulant) coated tubes by 10 ml syringe in two season summer (June) and winter (December). For the estimation of hormonal and cytokine level blood samples were centrifuged at 3000 rpm for 20 minutes to collect plasma. Plasma was collected and stored at -20°C to further hormonal analysis. Blood was processed for isolation of peripheral blood mononuclear cells (PBMCs).

ELISA of melatonin

Peripheral melatonin concentration in plasma was measured for all the samples with the help of a commercial kit (USCN, Life Sciences Inc., USA E90908Ge) following the manufacturer's instructions. The lowest limit of detection for plasma melatonin was 4.81 pg/ml. Intra and inters assay variations were 10% and 12%, respectively.

Isolation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated by density gradient centrifugation and an adapted method of isolating mononuclear cells by Boyum [11] also published elsewhere [12]. Lymphocyte separation media was used according to the manufacturer's instruction (HiSep™ LSM 1084, HiMedia, Mumbai, India). Briefly, the white band at the plasma-ficoll (Hisep, Himedia) interphase was collected and washed twice with PBS and finally suspended in complete media RPMI 1640 (Himedia, India) supplemented with 10% fetal bovine serum (FBS) and 100 units of penicillin and streptomycin (Sigma Aldrich, USA).

MTT assay PBMCs proliferation

Cell-mediated immune response was assessed by measuring PBMC proliferation in response to the T-cell specific mitogen, Concanavalin-A (Con-A), using a colorimetric assay based on the reduction of tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were counted using a hemacytometer and viability was determined by the trypan blue exclusion method. Viable cells (95%) were re-suspended in the complete culture medium, and 1 x 10^6 cells/ ml were adjusted per well in flat-bottom 24 culture well plate. T-cell specific Concanavalin-A (Con A, Sigma-Aldrich, St. Louis, USA) was added to the culture medium at the concentration of 5 µg/ml. Plates were incubated at 37°C with 5% CO_2 for 48h. Four hours before the completion of the 48h adding of 100 **µl of MTT (SRL, Bombay, India; 5** mg/ml in complete media RPMI-1640) was done per well. At 48h, 1 ml of acidified propanol (0.04 mol/l HCl in isopropanol) was added to each well and the optical density (OD) of each well was determined with a microplate reader (ELx-800, Biotek Instruments, Winooski VT, USA) equipped with a 570 nm wavelength filter. Mean OD values for each set of triplicates were used in subsequent statistical analysis. The response was calculated as the percent stimulation ratio (% SR) representing the ratio of absorbance of mitogen-stimulated cultures to control cultures:

$$\% \text{ Stimulation ratio (\%SR)} = \frac{\text{Optical density of Challenged (Con A)}}{\text{Optical density of Basal}} \times 100$$

Peripheral cytokine levels

Estimation of circulatory IL-2 level

Sandwich ELISA was carried out to quantify IL-2 level in plasma in triplicate according to the manufacturer's instruction (Immuno-

tech, Marseille Cedex, France). Intra-assay variation was between 3.3% and 7.2%, inter-assay variation was between 6.2% and 8.2%, sensitivity was 5 pg/mL.

Estimation of circulatory IL-6

Sandwich ELISA was carried out to estimate IL-6 level in plasma in triplicate according to the manufacturer's instruction (Koma Biotech, Seoul, Korea; Cat. No. K0331230). Lower and upper limits of analytic sensitivities were 16 and 1000 pg/mL.

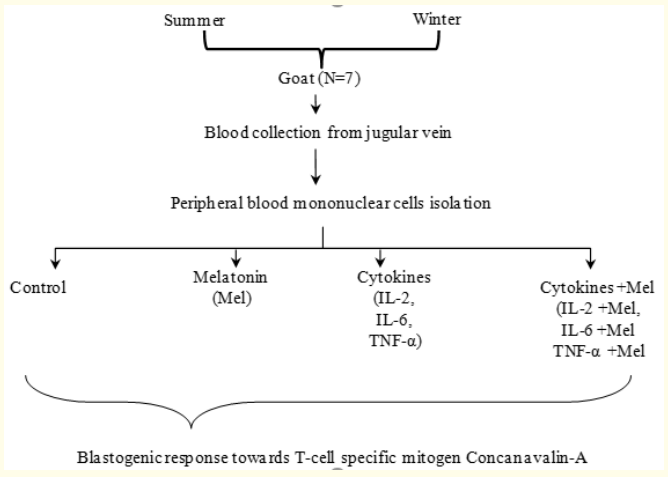
Estimation of circulatory TNF-α

Sandwich ELISA was performed to estimate TNF-α level in plasma in triplicate according to the manufacturer's instruction (Koma Biotech; Cat. No. K0331186). Lower and upper limits of analytic sensitivities were 16 and 2000 pg/mL.

Cytokine supplementation *in vitro* study

Melatonin (Sigma Aldrich, USA) and cytokines (rIL-2- Cat. No. RPA073Cp01; rIL-6-RPA079Cp01and TNF-α- RPA133Cp01from USCN, Business Co., Ltd, USA was purchased and a working stock of melatonin (500 pg/mL) and cytokines (50 pg/mL for IL-2, IL-6 and 10 ng/mL) were freshly prepared in complete media.

The experimental design is as follows.



Figure

Statistical analyses

To assess the difference between means for all parameters, one way ANOVA followed by Tukey's (honest significant difference) post-*hoc* test was performed using SPSS 17 INC., USA. Further, to show a comparison between summer and winter conditions, Student's t-test was performed. The values were expressed as mean ±standard error. All differences were considered statistically significant if $p < 0.05$.

Results

Peripheral cytokines level

The cytokines level was observed during both season summer and winter in plasma samples and a significant ($p < 0.01$) increase in the level of IL-2 and IL-6 and a significant ($p < 0.01$) declined level of TNF-α was noted during winter season compare to summer (Figure 1a-1c).

Melatonin estimation

The melatonin levels were estimated in plasma samples during the summer and winter seasons. A significant increase ($p < 0.01$) level of melatonin was observed during the winter season compared to summer (Figure 1d).

Cell-mediated immune function

Blastogenic response (% stimulation ratio) of peripheral blood mononuclear cells (PBMCs)

The cell-mediated immune responses of PBMCs were checked in terms of % stimulation ratio of PBMCs following T-cell specific mitogen concanavalin-A challenge. A significant ($p < 0.01$) increased %SR of PBMCs was observed during the winter season compared to summer (Figure 1e).

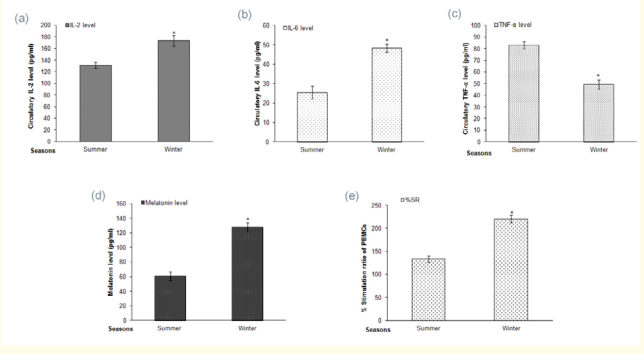


Figure 1: Seasonal variation of circulatory IL-2 (a), IL-6 (b), TNF-α (c) and circulatory melatonin level (d). Blastogenic response of PBMCs (e) in goat Capra hircus during summer and winter season. Data represents mean ± SEM; N = 7 vertical bar represents error bar. The significance of difference *$p < 0.01$; summer vs. winter.

Effect of recombinant cytokines on PBMC proliferative responses rIL-2 supplementation

Melatonin supplementation significantly ($p < 0.01$) enhances the proliferative response (%SR) of PBMC than control. No signifi-

cant effect of rIL-2 treatment was observed on %SR while co-treatment of rIL-2 with melatonin significantly ($p < 0.01$) increased %SR compared to rIL-2 group in both summer and winter (Figure 2a).

rIL-6 supplementation

No significant effect of rIL-6 was noted on %SR compare to control while melatonin co-supplementation with rIL-6 significant ($p < 0.01$) increased %SR compared to rIL-6 group in both summer and winter season (Figure 2b).

rTNF-α supplementation

Supplementation of rTNF-α significantly ($p < 0.01$) declined %SR of PBMCs compare to control. Melatonin co-treatment with rTNF-α significantly ($p < 0.01$) increased %SR compared to the rTNF-α alone group (Figure 2c).

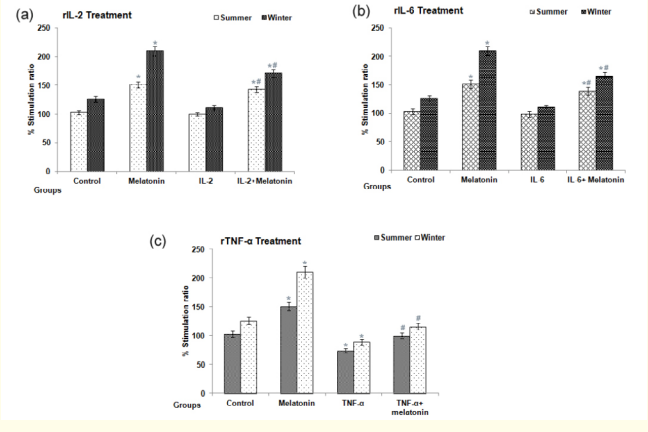


Figure 2: Effect of rIL-2 (a), rIL-6 (b) and rTNF-α (c) alone and in combination with melatonin on proliferative response of PBMCs of goat Capra hircus during summer and winter season. Data represents mean ± SEM; N = 7 vertical bar represents error bar. The significance of difference *$p < 0.01$; control vs. other groups and #$p < 0.01$; recombinant cytokine vs. cytokine + melatonin group.

Discussion

The present study demonstrates the effect of seasonal variation of melatonin induced circulatory cytokine levels and its plausible effect on the proliferative responses of immunocompetent peripheral mononuclear cells. Further, the effect of recombinant cytokines alone and in combination with melatonin elucidated its induced proliferative response on PBMCs during different seasons i.e. summer and winter. Higher levels of circulatory melatonin, IL-2, IL-6 along with increased proliferative response were observed during the winter season in goat. The immunostimulatory effect of recombinant rIL-2 and rIL-6 *in vitro* was additive with melatonin while the inhibitory effect of rTNF-α alone was observed on PBMCs. Melatonin supplementation was immunostimulatory for rTNF-α mediated decline in %SR of PBMCs.

The photoperiod influences the synthesis and secretion of melatonin [13]. The immunomodulatory effect of pineal melatonin has been widely studied in different seasonal animals [14] but less attention has been given to small ruminants like goats. A daily variation in melatonin level has been observed in goat during different seasons [12,15]. Most studies were focused upon the regulation of the reproductive activity of goats by melatonin [16,17]. In the present study, a high endogenous level of melatonin was noted during the winter season which could be due to a longer duration of the night than summer seasons. The high and longer duration melatonin might have increased proliferative response of PBMCs during winter. High melatonin has also been suggested to diminish free radical load in the physiological system by directly scavenging them and inducing antioxidant enzymes activity which we have previously suggested in a goat in a season and sex-dependent manner [12].

Cytokines are small molecular weight proteins synthesized and secreted by various immune cells [7] and act in a coordinated manner for appropriate immunological responses. High melatonin level was noted during the winter season that influenced IL-2 and IL-6 level positively and TNF-α level negatively to modulate the immunological functions of the goat. Melatonin has been suggested to act on T-lymphocytes by auto and paracrine mechanism to induce IL-2 which might help in the proliferation of the immune cells [18-20]. Further, circulatory IL-2 level also showed a pattern being parallel to melatonin suggesting that its production is being regulated by melatonin [21]. Further, IL-6 acts as a pleiotropic molecule in the regulation of various physiological processes of immune regulation like an inflammatory response [22]. T-cells and monocytes are the major sources of IL-6 which help in maturation of B-cells that also play a vital role in the regulation of the immune system [5,22,23]. Melatonin induced IL-6 during the winter season might regulate immune functions by increasing the responsiveness of T-cells towards IL-2. In our observation increased IL-6 level was noted during the winter season that might be due to increased melatonin level. Our result is in line with the finding of Ha., *et al*. [24] suggesting that melatonin *via* its membrane receptor (MT1/MT2) increases the level of IL-6. The increased IL-6 level induces the production of IL-2 from T-cells and also regulates the production and maturity of T-cells in presence of confined levels of

TNF-α in the physiological system [25]. TNF-α has been proposed to be an immunoregulatory molecule that can alter the balance of T regulatory cells. However, under the *in vitro* condition, TNF-α exerts a suppressive effect on immune cells [26]. The declined %SR of PBMCs in rTNF-α supplemented group might be due to the activation of the cellular death due to the presence of a high level of TNF-α [26]. Melatonin has been suggested to inhibit TNF-α secretion in a dose-dependent manner from PBMCs in humans [27] and also inhibits TNF-α mediated leukocytes and lymphocytes apoptosis [28].

Conclusion

In summary, melatonin might act as a key synchronizer in the regulation of seasonal immune responses by balancing different cytokines and their effects on cell-mediated immune responses. We observed that high melatonin causes an increased level of IL-2 and IL-6 and down-regulates TNF-α during the winter season. Melatonin significantly influences the proliferative responses of PBMCs in presence of rIL-2, rIL-6 and rTNF-α by enhancing the immunomodulatory effect of rIL-2 and rIL-6 and by diminishing immunosuppressive effect of rTNF-α in goat. In conclusion, we may propose that melatonin maintains immune-homeostasis by modulating the level of circulatory cytokines during different seasons. Melatonin might influence the secretion of immunomodulatory cytokine (i.e. IL-2 and IL-6) and also regulates the immunoinhibitory action of TNF-α to modulate cell-mediated immune function. Therefore, we may propose that melatonin might be responsible for synchronizing the synthesis and secretion of different cytokines to influence the immune responses of the animals during a different season.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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