Melatonin and the Enhancement of Immune Responses in Immature Male Chickens

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ABSTRACT Understanding the role of melatonin in affecting different physiological functions, especially immune responses, is becoming increasingly important in the basic and applied sciences. Enhancing the immune response will result in increasing disease resistance and, therefore, improve production efficiency. The purpose of the present study was to investigate the effects of melatonin, administered during the light or dark period, on BW, feed consumption (FC), and immune responses of immature chickens. Eight-week-old Cornell White Leghorn males were used in this study. The doses of melatonin were 0, 5, 10, 20, and 40 mg/kg BW. Melatonin was administered s.c. every 24 h for 7 consecutive d. The chicks were randomly divided into two groups; one group received injection during the middle of the light period, and the other group received injection during the middle of the dark period. All birds received 16 h light and 8 h darkness during a 24-h period. Body weights were measured before and after melatonin treatment, and FC was also measured. After the seven injections, blood samples were collected from the brachial vein, and total white blood cell (WBC) counts, differential cell counts, and activities of T and B lymphocytes were measured. Body weight was not significantly affected by dose of melatonin or time of injection. Furthermore, melatonin did not significantly affect FC; however, FC was significantly lower in the group that was injected in the dark vs. light period. The WBC counts of birds injected with 40 mg melatonin/kg BW were significantly higher than the WBC counts of saline-injected birds. The heterophil/lymphocyte (H/L) ratios of birds injected during the light period were significantly higher than those of birds injected during the dark period. T- and B-lymphocyte proliferation were significantly higher in birds injected with 40 mg melatonin/kg BW compared to saline-injected birds. These results indicate that melatonin in vivo is important in enhancing not only circulating WBC but also activities of B and T lymphocytes of immature male chickens without adversely affecting BW.

(Key words: chicken, melatonin, body weight, feed consumption, immunity)

INTRODUCTION

Melatonin is secreted during darkness by the pineal gland and sets the internal biological clock that governs different daily and seasonal cycles or rhythms in various physiological systems, including the cardiopulmonary, reproductive, excretory, thermoregulatory, behavioral, immune, and neuroendocrine systems in birds (Pang et al., 1996). Melatonin has different physiological functions in the body; it can affect feed consumption, energy metabolism, and immune responses. Bermudez et al. (1983) found that following melatonin injection, feed intake in domestic fowl was reduced. On the contrary, Apeldoorn et al. (1999) reported that melatonin supplemented in the diets of chickens had no effect on feed intake. This contradiction could be due to a difference in photoperiod used, dosage of hormone, or the type of bird used. In studying the effect of melatonin on energy metabolism, Apeldoorn et al. (1999) found that there were no significant interactions between lighting schedule and melatonin on energy metabolism in chickens. However, they found that the heat production related to activity was reduced when melatonin was supplemented to the diet.

The effects of melatonin on immune responses have also been investigated. In rats, melatonin has been shown to inhibit the development of mammary tumors (Tamarkin et al., 1981; Kothari et al., 1997). Melatonin added to the drinking water of quail resulted in an

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Abbreviation Key: FC = feed consumption; H/L = heterophil/lymphocyte; IFN-γ = interferon-γ; WBC = white blood cell.
increase in total white blood cells (WBC), an increase in the percentage of lymphocytes, a decrease in the percentage of heterophils, and a decrease in the heterophil/lymphocyte (H/L) ratio (Moore and Siopes, 2000). In Syrian hamsters, chronic daily administration of melatonin increased cytokine production (Champney et al., 1998) and splenic lymphoproliferative responses to concanavalin A (Champney et al., 1997). Previously, we reported that melatonin in vitro enhanced the mitogenic response of T and B lymphocytes in chickens (Kliger et al., 2000).

Length of photoperiod also affects immune responses. Moore and Siopes (2000) found that decreasing the photoperiod significantly increased lymphocyte percentage, decreased heterophil percentage, and decreased the H/L ratio in quail. A review of studies that examined the effects of photoperiod on immune function showed that short photoperiod enhances immune function in a variety of species (Nelson and Demas, 1996).

It seems that the effect of melatonin may depend on the time of administration. In chickens, Skvarlo-Sonta et al. (1991) found that the effect of exogenous melatonin on the diurnal rhythm of total WBC and serum agglutinins is more dependent on time of treatment than on the dose of hormone used.

It is clear that more studies are needed in chickens to further investigate the effects of melatonin on immune responses. Therefore, the present study was conducted to examine the effects of different doses of melatonin administered at different times on immune parameters as well as on BW and feed consumption (FC).

### MATERIALS AND METHODS

#### Animals and Experimental Design

Eight-week-old Cornell White Leghorn male chickens were used in this study. The chickens were housed in batteries and feed and water were supplied ad libitum. The same light treatment was provided for all the birds, 16 h light:8 h darkness, with lights on at 0600 h. The birds were randomly divided into two groups of 40 birds each. One group was injected with different doses of melatonin in the middle of the light period (Light group), the other injected in the middle of the dark period (Dark group). The 40 birds from each group were divided into five subgroups (two replicates each) and received daily s.c. injection with different dosages of melatonin dissolved in absolute ethanol. The doses were: 0, 5, 10, 20, and 40 mg melatonin/kg BW and were injected for 7 consecutive d. Individual BW, at the beginning and end of the experiment, and feed consumption per replicate per week were measured. After the last injection, blood was taken from the brachial vein, and the immune parameters were measured in the blood samples as described below.

### Immunological Parameters

#### Total WBC

Total WBC counts were made using brilliant cresyl blue dye (Haddad and Mashaly, 1990).

#### Differential Cell Counts

Differential counts were prepared using Hema 3 stain. The different types of WBC were counted, and percentages of heterophils and lymphocytes were calculated. Heterophil/lymphocyte ratio was also calculated.

#### Proliferation Assay for T and B Lymphocytes

The mitogen cell-proliferation assay used was described previously (Kliger et al., 2000). Briefly, leukocytes were separated from red blood cells using histopaque-1077. Leukocytes were plated at 5 × 10⁵ cells per well, and 50 µL of Con A at 12.5 µg/mL or 50 µL of pokeweed mitogen at 25 µg/mL was added.

Cells were then incubated at 37 C in a humidified atmosphere of 5% CO₂ for 48 h. One µCi of ³H-thymidine was added to each well. Cells were again incubated at 37 C under 5% CO₂ for 18 h to allow for ³H-thymidine uptake. Cells were harvested onto glass-fiber filters using a cell harvester. The filters were placed in scintillation vials with scintillation fluid and were counted using a scintillation counter. Counts per minute were determined for each well. Control counts from wells with no mitogen were subtracted from treatment counts.

### Statistical Analysis

SAS® software general linear models procedure (SAS Institute, 1996) was used to analyze data with two-way ANOVA with dose of melatonin and time of injection as the main effects. Means were separated using Duncan’s multiple-range test with significance set at P < 0.05.

### RESULTS AND DISCUSSION

Overall, the change in BW was not significantly affected by dose of melatonin or time of injection (data not shown). These results are similar to those of Apeldoorn et al. (1999) who found that melatonin in broiler feed did not affect BW. However, the results are different from those of Injidi and Forbes (1983) who found that melatonin inhibited growth of 1-d-old male chicks when they were injected s.c. for 28 d. Differences in the results could be due to age of birds used (1 d vs. 9 wk) and duration of injection (7 d vs. 28 d). Furthermore, Clark and Classen (1995) found that adding melatonin to the diets of broiler chicks inhibited growth between 0 to 14 d of age.

In our study, we found no effect of melatonin on FC. These results agree with those of Apeldoorn et al. (1999) who showed that adding melatonin to a broiler diet...
did not affect feed intake. Other researchers found that melatonin inhibited FC (Bermudez et al., 1983; Clark and Classen, 1995). Differences in results could be due to the age of birds used and duration of treatment. In the present study, even though different doses of melatonin did not affect FC, the birds that were injected during the dark period consumed significantly less feed than birds injected in the light period (304 g/bird per wk light period vs. 263 g/bird per wk dark period). This reduction in FC could be caused by an increase in melatonin levels due to an additive effect of exogenous and endogenous melatonin during the dark period compared to the light period. Endogenous melatonin is higher at night compared to during the day (Leung, 1991).

Data for the total WBC counts and T- and B-lymphocyte activities are shown in Figures 1, 2, and 3, respectively. Melatonin was found not only to significantly increase total WBC but also to significantly increase T- and B-lymphocyte activities. The overall total WBC of birds injected with 40 mg melatonin/kg BW were 90.5 × 10⁶/mL compared to 70.7 × 10⁶/mL for saline-injected birds. The counts per minute for T-lymphocyte proliferation of birds injected with 40 mg/kg BW were 48,308 vs. 10,652 for saline-injected birds, whereas the counts per minute for B-lymphocyte proliferation of birds injected with 40 mg/kg BW were 14,060 vs. 4,279 for saline-injected birds. These results indicate the importance of melatonin administered in vivo on enhancing the proliferative activity of T and B cells assayed in vitro. These results reemphasize our previous findings in chickens in which we found that melatonin in vitro enhances the activities of T and B lymphocytes (Kliger et al., 2000). Furthermore, melatonin receptors were found in the chicken bursa of Fabricius (Liu and Pang, 1993) and duck thymus (Liu and Pang, 1992). B and T lymphocytes are involved in humoral and cell-mediated immunities. Enhancing these immunities might assist in increased disease resistance.

It seems that melatonin is not only important in chickens but has also been reported to be an immunomodulator in mammalian species as well. Champney et al. (1997), using Syrian hamsters, found that s.c. injection of melatonin increased splenic lymphoproliferative responses to concanavalin A. Furthermore, Caroleo et al. (1992) determined that melatonin injected s.c. restored the impaired T-helper cell activity in immunodepressed mice. Giordano and Palermo (1991) showed that melatonin in vivo enhanced the capacity of murine splenocytes to mediate antibody-dependent cellular cytotoxicity. The immunomodulatory role of melatonin could be due to the presence of binding sites in blood lymphocytes from humans; granulocytes, thymus, and spleen from rodents; and bursa of Fabricius from birds (Calvo et al., 1995).

The melatonin influence on immune responses could be through the stimulation of cytokine production (Mae-
stroni, 1995), which in turn enhances lymphocyte activities. Garcia-Maurino et al. (1997) suggested that melatonin in vitro enhanced production of interleukin-2, interleukin-6, and interferon-γ (IFN-γ) by human peripheral blood mononuclear cells and Colombo et al. (1992) determined that IFN-γ was produced in vitro by mice splenocytes after melatonin stimulation. Furthermore, Champney et al. (1998) found increased serum IFN-γ levels in Syrian hamsters after melatonin injection. Different cytokines are involved in the growth and activation of different mononuclear cells (Cruse and Lewis, 1995). It has also been suggested that melatonin influences the immune response through opiateergic mechanisms (Maestroni et al., 1987).

Moore and Siopes (2000) found that total WBC counts in Japanese quail increased significantly during a shorter photoperiod compared to a longer photoperiod and constant lighting, indicating that darkness increases total WBC numbers, which could be due to increased melatonin levels in darkness as reported by Leung (1991). However, in our study, we found that total WBC counts were significantly reduced during darkness. This reduction cannot be explained physiologically.

In general, different doses of melatonin did not affect the percentage of heterophils or lymphocytes or the H/L ratio (data not shown). However, there were significant differences in cell type percentages and H/L ratios between the light and dark periods (Table 1). The percentage of lymphocytes was highest during the dark period, and the percentage of heterophils was lowest during the dark period. The H/L ratio was significantly lower during the dark period compared to the light period. Our results are in agreement with those of Moore and Siopes (2000), who found that decreasing the photoperiod or adding melatonin to the drinking water of Japanese quail significantly increased lymphocyte percentage, decreased heterophil percentage, and decreased the H/L ratio. They concluded that constant lighting was more stressful than shorter daily photoperiods, as indicated by the H/L ratio, and that exogenous melatonin has an immuno-reconstituting effect on quail placed in constant lighting.

In summary, our results demonstrate that melatonin in vivo is important in enhancing not only circulating WBC but also activities of B and T lymphocytes of immature male chickens. In addition, melatonin may also reduce stress, as indicated by the reduction of the H/L ratio during the dark period.

### REFERENCES


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### TABLE 1. Effect of time of melatonin injection for 7 consecutive d on percentage heterophils, lymphocytes, and heterophil/lymphocyte (H/L) ratio in immature male White Leghorn chickens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lymphocyte</th>
<th>Heterophil</th>
<th>H/L ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>56.1b</td>
<td>28.3a</td>
<td>0.532a</td>
</tr>
<tr>
<td>Dark</td>
<td>64.8a</td>
<td>23.7b</td>
<td>0.391b</td>
</tr>
</tbody>
</table>

a,bMeans within a column and with no common superscript differ significantly (P < 0.05); n = 33 to 34.