TIME DEPENDENT IMMUNOMODULATORY RESPONSE OF EXOGENOUS MELATONIN TO KILLED *PASTEURELLA MULTOCIDA* (P$_{52}$ STRAIN) VACCINE IN ALBINO RATS

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Abstract: The effect of time of administration of exogenous melatonin (M) at the rate of 100 µg/Kg BW of rat/day for 14 days on immunomodulation to killed *Pasteurella multocida* (P$_{52}$ strain) vaccine (KPMV) was investigated in male albino rats during spring season with photoperiod of LL 13: DD 11 h and 25 ± 2.5°C air temperature and 70 ± 4% relative humidity. The experiment was conducted at an altitude of 172 mts above mean sea level at latitude 28.20° north, longitude 79.24° east (Bareilly, U.P. India). The experimental animals were divided in groups of 8 rats each, as KPMV + M at 4.00 h; KPMV + M at 16.00 h; KPMV and their controls M4, M16, PBS respectively. Humoral immune response was monitored at weekly intervals by an indirect ELISA and cellular immunity by leukocyte migration inhibition test (LMIT) and delayed type of hypersensitivity (DTH). As evinced by in-vitro assays and in-vivo protection studies, both humoral and cellular immune responses to KPMV were augmented in rats receiving exogenous melatonin at 4.00 h as compared to slightly reduced responses in rats treated with melatonin at 16.00 h. It was concluded that the circadian timings of melatonin administration modulate immune response in rats.

Key words: time dependence immunomodulation melatonin humoral immune response cell-mediated immune response circadian

INTRODUCTION

The neuroendocrine system modulates the functioning of immune system through neuropeptides and neurohormones (1), which supports the existence of a link between neuroendocrine and immune system (2). Of the different neuropeptides and neurohormones, melatonin, the pineal neurohormone has been reported to influence immune system (3). It has been demonstrated that the mice sensitized with
melatonin enhance antibody production against antigens, which are T-cell dependent (4, 6). Besides having effect on humoral immune response, melatonin also influences cell mediated immune function (7, 8). Exogenous melatonin can counteract the immunosuppression caused by acute stress or pharmacologically administered corticosteroids (9). Basically, pineal gland being a part of sensory system transduces external environmental circadian and circannual signals (day light and ambient temperature) into hormonal responses, which modulate neuroendocrine and immune functions, by circadian and rhythmic synthesis and release of melatonin from pineal gland (10). Maestroni and Pierpaoli (5) reported that the exposure of mice to constant artificial light for 3–4 generations impairs their growth rate and immune function. Apart from the effect of constant exposure to light, the immunomodulatory effects of exogenous melatonin in mice are also influenced by time of the administration of melatonin (5, 6, 11). Melatonin exerts its effect only on antigen activated immune cells \textit{in vivo} and has no immunological effect in unvaccinated animals (6, 8, 12).

Information is lacking on the immunomodulatory effect of exogenous melatonin, as assessed by \textit{in vivo} immunoassay and \textit{in vivo} challenge experiments in relation to the time of administration of melatonin. The research findings reported herein, focus on the time dependent effect of subcutaneously administered melatonin in the morning at 4.00 h and in the evening at 16.00 h on humoral and cellular immune response in rats inoculated with killed \textit{Pasteurella multocida} (P$_{52}$ strain) vaccine.

**MATERIALS AND METHODS**

The experimental animals, male albino rats and guinea pigs used in this study were obtained from the Laboratory Animal Resource Section, I.V.R.I., Izatnagar (U.P.). Melatonin and Histopaque 1077 were procured from Sigma chemical company, USA. Dulbecco’s Minimum Essential Medium used for lymphocyte culture was purchased from Gibco-BRL USA. Anti-rat IgG-HRPO conjugate used for ELISA was gifted by immunochemistry laboratory of National Institute of Immunology, New Delhi. The 96 well enzyme immunoassay plates were procured from Nunc, Denmark, whereas the 12 well LMIT plates were procured from LAXBRO (India).

The killed \textit{Pasteurella multocida} (P$_{52}$ strain) vaccine and virulent live \textit{Pasteurella multocida} (P$_{52}$ strain) were received from the National Referral Centre, Division of Standardization for Veterinary Biologicals, I.V.R.I., Izatnagar (U.P.).

**Experimental design:**

Sixty-four adult male albino rats, each weighing around 120 gms were reared in polypropylene cages during spring season with photoperiod of LL 13: DD 11 h and 25 ± 2.5°C air temperature and 70 ± 4% relative humidity. The experiment was conducted at an altitude of 172 mts above MSL (mean sea level) and located at latitude 28.20° north, longitude 79.24° east.

The rats were divided into four groups
of 16 rats each and treated as follows.

Group I: Of the 16 rats, 8 were vaccinated with 0.5 ml of KPMV subcutaneously (KPMV group) and another eight rats were injected with 0.5 ml of sterile phosphate buffer saline (PBS) and kept as control.

Group II: All the 16 rats were vaccinated. Out of these, 8 rats were injected subcutaneously with 0.2 ml of 100 fig of melatonin/Kg BW/day at 4.00 h (KPMV+M4 group) while remaining 8 rats were treated with melatonin similarly at 16.00 h (KPMV + M16 group).

Group III: Out of 16 unvaccinated rats 8 were injected with melatonin at 4.00 h (M4) and remaining 8 rats at 16.00 h (M16).

Group IV: Sixteen unvaccinated rats were divided in 2 groups and injected with PBS, 0.2 ml/rat/day at 4.00 and 16.00 h respectively for 14 days and were used as control for Group II and III.

Rats were vaccinated once at the start of experiment. From the same day both melatonin and PBS was administered daily as per the schedule given above. Melatonin was dissolved in few drops of ethanol and then diluted at required concentration in isotonic phosphate buffer saline. The melatonin was injected at given times for 14 days.

In vivo challenge experiment using virulent live Pasteurella multocida (P52 strain) was carried out on day 24th post vaccination. The rats were bled on day 0, 7, 14, 24 day post vaccination and day 3 and 5 post challenge. All the blood samples were processed for isolation of peripheral blood lymphocytes using histopaque and for collecting sera samples.

Ethical guidelines prescribed by Council of International Organization of Medical Sciences have been followed for care and management of the rats during study.

Humoral immune response

An indirect ELISA was performed for estimation of antibody titers against KPMV in various experimental groups of rats on day 0, 7, 14, 24 post vaccination, following the method of Engvall and Perlman (13) with some modifications. The test sera from individual rats, known-positive serum to KPMV and known-negative serum of rats were used for all the tests.

All the reagents were used in 50 µl volumes. The wells of ELISA plates were coated with purified sonicated preparation of KPMV at the rate of 2 µg/well in carbonate-bicarbonate buffer (pH 9.6) and incubated for 90 min at 37°C and washed thrice using PBS Tween-20. The unquenched sites in the well were blocked with 5% (w/v) defatted milk powder in PBS Tween-20. After washing the wells, two fold dilution (starting 1 : 25) of the test and control negative sera were added to plate in triplicate and allowed to react for 1 h at 37°C and then washed. Optimized dilution
(1:4000) of anti-rat HRPO conjugate was added to each well and incubated for 1 hour for 37°C and then washed. The tests were developed using OPD/H₂O₂ substrate prepared in phosphate citrate buffer (pH 5.0) and reaction was stopped by 1 M H₂SO₄. Optical densities were measured at 492 nm in an ELISA reader (ECIL, Hyderabad, MicroScan, MS5605A). The optical densities of the dilution of test serum that was double to that of control negative serum was considered as positive and thereby the criterion for determining the titers.

**Cellular immune response**

Lymphocyte migration inhibition test (LMIT) was carried out as described by Sorborg and Bendisen (14). Two ml of blood was collected in sterile test tube containing heparin (20 IU/ml of blood) and diluted in equal volume of RPMI 1640 (Sigma). Diluted samples were transferred to centrifuge tubes containing histopaque 1077 (Sigma) half of the volume of diluted blood sample and centrifuged at 1000 RPM for 45 minutes. Lymphocytes were collected by Pasteur pipette from the interphase of plasma and histopaque and were washed thrice with RPMI 1640 at 1000 RPM for 30 minutes. Lymphocytes were collected by Pasteur pipette from the interphase of plasma and histopaque and were washed thrice with RPMI 1640 at 1000 RPM for 30 minutes. The cell concentration was adjusted to 2×10⁸ cell/ml in RPMI 1640 with 5% fetal calf serum. The sterile glass capillaries were filled with cell suspension and then plugged at one end with plasticin and centrifuged at 800 RPM for 3 minutes. The capillaries were cut using a diamond pencil and placed in migration chamber (Laxbro) with the help of properly coated (as per the length of capillary) non-toxic silicon grease. Then the chambers were filled with growth medium containing H.S. killed antigen and growth medium without antigen. The plates were incubated overnight at 37°C. The area of migration was measured with the help of camera lucida fitted to microscope and percent of migration inhibition (MI) was calculated as:

\[
\text{Percent MI} = \frac{\text{Area of migration with antigen}}{\text{Area of migration without antigen}} \times 100
\]

**Production of lymphokines:**

Lymphocytes were isolated from the blood of rats from different groups viz. - KPMV, KPMV + M4, KPMV + M16, M4, M16 and PBS control for LMIT. The lymphocytes were cultured (approximately 10⁸ cells/ml) in vitro at 37°C for 24 h in presence of 20 μl/ml of KPMV. After incubation the supernatants were collected, centrifuged at 10,000 g for 10 min at 4°C and clear supernatants were stored at –20°C until used.

**Delayed type of hypersensitivity test:**

Nine KPMV sensitized and 3 unsensitised (PBS injected) guinea pigs were shaved for a small area on abdominal region. The sensitized and unsensitized animals were injected with lymphokines (at the rate of 0.5 ml) intradermally in the shaved abdominal skin. Skin reactions were read between 24 and 48 h. Then about 5 mm piece of skin from inoculated site of all the guinea pigs were collected and processed for histopathology.

**Statistical analysis**

The data obtained from ELISA and LMIT
were evaluated statistically by analysis of variance. ‘P’ value of <0.05 was taken as the level of significance.

RESULTS

The antibody response to KPMV as assessed by indirect ELISA in various experimental groups of rats as influenced by the exogenous melatonin and time of its administration is presented in Table I. The result shows that the rats in three different groups, which received either KPMV or KPMV + exogenous melatonin, seroconverted to the KPMV irrespective of time of administration of melatonin. However, the groups receiving either melatonin or PBS did not show antibody response to KPMV before virulent challenge. It is evident that the rats in KPMV + M4 group could elicit significantly (P<0.05) higher antibody response (3.35 ± 0.06) compared to rats in KPMV + M16 (2.80 ± 0.13) and KPMV (2.92 ± 0.09) group at all the serum sampling intervals post vaccination. The antibody response increased gradually and peaked at day 24 post vaccination. Following challenge on day 25, a slight fall in antibody titre was noted on day 3, but again increased on day 5 post challenges. The groups receiving either melatonin or PBS at both 4:00 h and 16:00 h, slightly seroconverted (antibody titre in the range of 1.15 ± 0.06 and 1.12 ± 0.07) to Pasteurella multocida challenge on day 3. The rats receiving either KPMV or both KPMV and melatonin showed slight fall in antibody titre on day 3, but increased significantly (P<0.05) in KPMV + M4 (3.65 ± 0.18) group compared to KPMV + M16 (2.85 ± 0.09) and KPMV (3.15 ± 0.09) group on day 5th post challenge.

The cell mediated immune response as monitored by LMIT increased gradually from day 7 to day 24 post vaccination in all the groups receiving either KPMV or both KPMV and melatonin, whereas the control groups did not show any significant response (Table II). On day 24 post vaccination KPMV + M4 group showed significantly (P<0.05) higher (28.42 ± 1.89)

TABLE I: Effect on humoral immune response in the rats (n=8) inoculated with KPMV and its modulation by melatonin administration.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post immunization</th>
<th>Days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Control</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>M4</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>M16</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>KPMV</td>
<td>1.74±0.22</td>
<td>2.45±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KPMV+M4</td>
<td>1.65±0.10</td>
<td>2.90±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KPMV+M16</td>
<td>1.72±0.16</td>
<td>2.40±0.16&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a, b, c superscripts differ significantly (P<0.05) in a column, D- all rats died post challenge, values in parenthesis indicate number of animals survived after KPM challenge.
percentage of leukocyte migration inhibition, compared to KPMV+M16 (24.00 ± 1.82) and KPMV (25.92 ± 2.01) groups. The control groups receiving either melatonin or PBS showed inhibition in the range of 15.00 ± 2.56 and 17.01 ± 2.58 throughout the experiment. Following the virulent challenge on day 25, the cellular immune response decreased on day 3 and increased on day 5 post challenge, registering significantly (P<0.05) higher percent inhibition in KPMV + M4 (31.75 ± 3.16) group as compared to KPMV + M16 (27.45 ± 2.06) and KPMV (29.56 ± 2.78).

The results of histopathology of skin of KPMV primed guinea pigs from lymphokine injected area after 48 h of delayed type of hypersensitivity reaction are shown in figures 1, 2, 3, 4. The KPMV + M4 group of rats showed infiltration of large number of mononuclear cells, polymorphs and macrophages at 48 h post lymphokine injection (Fig. 2). However, the rats in KPMV + M16 group showed comparatively few mononuclear cells, polymorphs and macrophages (Fig. 3). On the other hand, the rats in KPMV group showed marked infiltration of polymorphs, macrophages as well as mononuclear cells (Fig. 4). The rats in control groups (M4, M16 and PBS) did not show any cellular infiltration (Fig. 1).

The protection conferred to Pasteurella multocida (P 52 strain) virulent challenge in various groups of rats vaccinated with either KPMV or treated with melatonin is shown in Table III. All the rats in KPMV + M4 group survived the challenge, however, 25% of rats in each of KPMV + M16 and KPMV

| Table II: Effect on cell-mediated immune response in the rats (n = 8) inoculated with KPMV and its modulation by melatonin administration. |

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post immunization</th>
<th>Days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 7 14 24</td>
<td>3 5</td>
</tr>
<tr>
<td>Control</td>
<td>15.00±2.56</td>
<td>15.52±1.72</td>
</tr>
<tr>
<td>M4</td>
<td>16.50±2.18</td>
<td>16.88±1.59</td>
</tr>
<tr>
<td>M16</td>
<td>15.52±1.23</td>
<td>15.88±1.59</td>
</tr>
<tr>
<td>KPMV</td>
<td>16.58±1.56</td>
<td>20.53±2.01</td>
</tr>
<tr>
<td>KPMV+M4</td>
<td>17.58±2.14</td>
<td>22.42±1.56</td>
</tr>
<tr>
<td>KPMV+M16</td>
<td>17.07±2.19</td>
<td>18.00±1.56</td>
</tr>
</tbody>
</table>

a, b, c superscripts differ significantly (P<0.05) in a column, D- all rats died post challenge, values in parenthesis indicate number of animals survived after KPM challenge.

| Table III: Percent survival of rats (n = 8) to virulent Pasteurella multocida challenge in different groups. |

<table>
<thead>
<tr>
<th>Days post challenge</th>
<th>KPMV+M4</th>
<th>KPMV+M16</th>
<th>KPMV</th>
<th>M4</th>
<th>M16</th>
<th>PBS control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>3</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td>37.5%</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>100%</td>
<td>100%</td>
<td>75%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>5</td>
<td>100%</td>
<td>75%</td>
<td>75%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Fig. 1: Micrograph (H & E stain, 400X) of skin of guinea pig inoculated with lymphokines form PBS group of rats showing no detectable cellular changes at 48 hour.

Fig. 2: Micrograph (H & E stain, 400X) of skin of guinea pig inoculated with lymphokines form KPMV+M4 group of rats showing infiltration of large number of mononuclear cells, polymorphs and macrophages at 48 hour.

Fig. 3: Micrograph (H & E stain, 400X) of skin of guinea pig inoculated with lymphokines form KPMV+M16 group of rats showing few mononuclear cells, polymorphs and macrophages at 48 hour.

Fig. 4: Micrograph (H & E stain, 400X) of skin of guinea pig inoculated with lymphokines form KPMV group of rats showing marked infiltration of mononuclear cells, polymorphs and macrophages at 48 hour.
groups succumbed to death on day 5 and 4 respectively. The level of protection in M4 group was higher up to day 3rd post challenge than M16 and PBS treated groups but all the rats in these control groups died before the termination of experiment on 5th day.

**DISCUSSION**

Time dependent effect of exogenous melatonin on the immune cells has been reported in mice (5). The immunomodulatory role of melatonin in inbred mice (BALB/c) show that, 10 ug of melatonin/kg B.W. administered at 8.00 h in the morning did not produce any effect on the primary immune response to sheep RBC (SRBC). In contrast, an immune enhancing effect was induced by the same close administered in the evening at 16.00 h. The author of the study suggested that the stimulatory effect on cellular and humoral immune response in BALB/c mice may depend on the release of immunoenhancing opioid agonist (endorphin, dynorphin and Leu-enkephalin) or on the appropriately timed melatonin injections (5). Binding of [I^{125}] melatonin by T lymphocytes exhibited properties such as dependence on time and high affinity binding sites and temperature (15, 16). Lopez-Gonzalez (15) reported that increasing concentration of native melatonin inhibited binding of melatonin on T lymphocytes, probably recognizing the melatonin binding sites on T lymphocytes by circulating melatonin. These studies on binding of [I^{125}] melatonin to T lymphocytes showed inverse correlation with the nocturnal melatonin production.

In the present study, administration of melatonin for 14 days in the morning at 4.00 h (when endogenous melatonin peak starts declining (17, 18), in albino rats primed with KPMV showed increase in antibody as well as cellular immune response. It indicates that the morning 4.00 h administration of melatonin has immunostimulatory effect in vaccinated rats. This immunostimulatory effect of melatonin at 4.00 h can be explained on the basis of duration versus coincidence hypothesis (19). The morning (4.00 h) subcutaneous administration of melatonin coincides with decreasing endogenous melatonin level. It prolongs the duration of circulating melatonin period of circadian rhythm, which may synchronize with the sufficient number of antigen-activated lymphocytes, which produce stimulatory effect on humoral and cellular immune response. However, evening (16.00 h) administration of melatonin caused decrease in humoral and cellular immune response, marginally, compared to group receiving KPMV only. Probably, administration of melatonin at 16.00 h might have raised the total blood melatonin level by coinciding with the nighttime increase in endogenous melatonin level. This unusually higher level of melatonin might have caused marginal suppression in the immune response by down regulation of its receptors on immune cells (21, 22). It is to mention that our findings are not in agreement with Maestroni et al. (5), where they have demonstrated that evening (16.00 h) administration of melatonin has immunostimulatory effect. It is speculated that geographical location may be a cause of this variation, and it is known that geomagnetic element, in terms of EMF influence the secretions activity of pineal melatonin secretion. Further, they suggested that low dose of melatonin (10 ug/kg B.W.) produce significant depression of the primary immune response to SRBC. However, 25% higher protection shown by
control group against MI6 group on day 2 (Table III) is anomalous and may be due to unknown reasons.

Further, the results of in vitro assay for humoral and cellular immune response to KPMV with variable melatonin treatments are in agreement with in vivo challenge experiment and histopathology of skin obtained from guinea pigs subjected for DTH testing. Therefore, we infer that, exogenous melatonin administered in albino rats at 4.00 h causes immunoenhancing effects whereas its administration at 16.00 h leads to slightly depressed immune responses. It is suggested that the estimation of total serum melatonin level, at each blood-sampling interval be required to conclude determinative immunomodulatory effect of melatonin.

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