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Role of Melatonin in Modulation of Immune Status of Pregnant Female Indian Short Nosed Fruit Bat *Cynopterus* sphinx

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Abstract

Pregnancy is associated with profound immunological changes that are characterized by a strong activation of certain components of the innate immune defense and a down-regulation of adaptive immune functions. This shift in balance of the immune system towards an innate dominance is thought to be important for the maintenance of pregnancy. Based on our observation in the short nosed fruit bat Cynopterus sphinx, a seasonal breeder, we show for the first time that melatonin injection to the pregnant females significantly increases lymphocyte proliferation of spleen and consequently the circulating level of lymphocytes and percent stimulation ratio of splenocytes, thereby improving immune status during pregnancy. We have reported earlier that during pregnancy melatonin level increases significantly which in turn might improve the maternal immunity. Towards establishing this inference we used a physiological dose of p-chlorophenylalanine (p-CPA), an indirect antagonist of melatonin, which reduced circulatory melatonin level and thereby reduced the immune status. It is conceived that specific immune adaptation is conveyed to the fetus through placental transfer of melatonin thereby controlling fetal immunity as well. This could be an adaptation during pregnancy to protect the mother from various external threats.

Keywords: Cynopterus sphinx, Immunity, Melatonin, Pregnancy

1. Introduction

Large physiological adjustments are required in the mother to successfully carry the pregnancy through. These changes result from signals passing between the conceptus (especially the trophoblast) and the mother. During this phase every system in the body is affected, which does include immune system. Immune system is part of a complex signaling system between cells that has developed the ability to recognize self and non-self but not required for the mother to cope with the fetus as an allograft[2]. Researchers studying pregnancy have found a relationship between the mother's immune system and the placenta. The placenta is rich in blood vessels reaching from the mother and, hence, fetus is protected via the mother's immune system. It appears that the placenta produces chemicals that alter the mother's immune system.

Pregnancy is associated with profound immunological changes that are characterized by a strong activation of certain components of the innate immune defense and a down-regulation of adaptive immune functions. This shift in the balance of the immune system towards an innate dominance is thought to be important for the maintenance of a pregnancy[3-5]. Pregnancy seems to strengthen humoral immunity and weaken cell-mediated immunity and, thus, helps the fetus to survive during pregnancy[6].

The mechanisms that mediate seasonal changes in physiological and behavioral processes also appear to regulate the seasonal changes in immunity of vertebrates. Individuals use photoperiodic information to initiate

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or terminate specific seasonal adaptations in order to maintain positive energy balance[7]. The secretory pattern of melatonin allows individuals to ascertain the time of the year and develop seasonally appropriate adjustments in energy use[8]. Generally, melatonin enhances immune function whereas glucocorticoids compromise it[9-11]. But there has been no report on the immune status during gestational period and the impact of melatonin in the nocturnal seasonal breeder Indian short nosed fruit bat, Cynopterus sphinx. Hence, in the present experiment we studied the immune status of pregnant female bats in relation to melatonin by using an indirect antagonist of melato-nin, p-chlorophenylalanine (p-CPA).

To check the immunity we have considered the hematological parameters ie., Total Leukocyte Count (TLC), and Lymphocyte Count (LC) of peripheral blood and the % stimulation ratio of splenocyte proliferation challenged with Concanavalin A (Con A) of pregnant female bat Cynopterus sphinx.

2. Materials and Method

2.1 Animals and Maintenance

One hundred and twenty pregnant female bats, Cynopterus sphinx (as judged by presence of sperm in vagina for initial days and later by feeling the abdomen; body mass; ~ 50 - 60g) were collected locally from the vicinity of Varanasi (Lat.25^0 1 8'N; long 83^0 l'E) during first week of April (the month when bats show normal embryonic development; NED). Bats were kept in wire such as guava, banana, papaya, melons, etc., and water ad libitum.

All animal experiments were conducted in accordance with institutional practice and within the framework of revised Animal (Specific Procedure) Act of 2002.

2.2 Experimental Design

The experiment was conducted during April month when environmental parameters were as follows: temperature maximum 26.4^{0}C to 28.6^{0}C, minimum, 12^{0}C to 13.7^{0}C; humidity maximum 85.8 to 69.7%, minimum 50.7 to 35.2%; day length 11.15 to 11.45 hours).

2.2.1 Group I- Sham Control (Treated with Normal Saline)

Forty pregnant female bats that received ethanolic saline (0.9% NaCl + 20 uL of ethanol) injection during evening hrs, within 5.00 to 6.00 pm, i.e., hrs before darkness and served as sham control. They were sacrificed as five each on day 15, 30, 45, 60, 75, 90, 100 in NED group of gestation and After Delivery (AD).

2.2.2 Group II- Melatonin Treated

Forty pregnant female bats received melatonin injection (25ug/0.1mL/100g Bwt/day) during evening hours, within 5.00 to 6.00 p.m. i.e., 1-1½ hr before darkness. They were sacrificed as five each on day 15, 30,45, 60, 75, 90,100 in NED group of gestation and After Delivery (AD).

2.2.3 Group III- Parachlorophenylalanine (p-CPA) Treated

Forty pregnant female bats received p-CPA (0.5mg//0.1mL/100g Bwt/day) till delivery during afternoon hours 14.30 to 15.30 hr). They were sacrificed as five each on day 15, 30, 45, 60, 75, 90, 100 in NED group of gestation and After Delivery (AD).

2.2.4. Preparation of Melatonin and p-CPA for Injection

Melatonin and parachlorophenylalanine (p-CPA) were purchased from Sigma- Aldrich Company, St. Louis, USA. Melatonin solution was prepared by dissolving it in a few drops (10 uL) of ethanol and then diluting it with 0.9% NaCl to the desired concentrations depending upon the doses to be given as per experimental requirement. p-CPA was first dissolved in a few drops of ethanol and then diluted to the desired concentration with 0.9% phosphate-buffered saline and brought to pH 6.0 by addition of 5 mMol l-^{1} Na_2HPO_4 and stored at 4 ^{0}C for daily use.

2.2.5 Hematological Parameters

Blood was taken in a WBC pipette and diluted 20 times in Turk's fluid (2.0 mL glacial acetic acid, 0.1 g mercuric chloride, one drop aniline, and 0.2 g gentian violet) and the white blood cells were counted (no/mm^3) in Neubauer's counting chamber (Spencer, USA) in a microscope. Thin film of blood was prepared and stained with Leishman's stain and lymphocytes were counted under oil immersion lens of Leitz MPV3 microscope.

2.2.6 Reagent and Culture Medium for Blastogenic Response

Tissue culture medium RPMI-1640 and all other chemicals were purchased from Sigma Chemical Co., St. Louis,

USA. The culture medium was supplemented with 100 U/mLl penicillin, 100 (ig/mL streptomycin, and 10% fetal calf serum. Spleen was processed for preparation of single cell suspensions. The number of cells was adjusted to 1 x 10^6 cells/mL in complete medium. Two milliliters of cell suspension was placed in duplicate culture tubes and kept at 37 °C in a 5% $C0_2$ incubator (Hera Cell, Heraus, Germany) for 72 h. Blastogenic response was measured in terms of [^{3}H]thymidine (specific activity 8.9 Ci/mM) uptake against stimulation by Con A of the splenocytes[12] and present as % stimulation ratio (% SR).

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

2.3 Sampling

During experiment five pregnant female bats of each group were sacrificed on day 15, 30, 45, 60, 75, 90, 100 in NED group of gestation and After Delivery (AD). The body weight of bats was noted. Spleen was dissected out, weighed on a monopan balance and processed for % SR estimation. The trunk blood was collected in heparinized tubes and centrifuged at 3000 x g to collect the plasma. The plasma was stored at -20 ^{0}C till hormonal analysis (melatonin, estradiol and progesterone). For the RIA of melatonin, subclavian vein blood (0.5 mL) was collected from each female during the night time under dim red light prior to the day of sacrifice.

2.4 Radioimmunoassay

RIA of estradiol was performed with the help of a commercial kit purchased from Leuco Diagnostic Inc., Miss., USA. The recovery and sensitivity for estradiol were 102.2% and 1-45pg/mL, respectively. Intra- and inter-assay variations of estradiol were 9.2% and 4.3%, respectively. Progesterone assay was done using commercial RIA kit from Binax, 217, Portland, Maine, USA. The sensitivity of the assay was 50 pg/tube and the intra- and inter-assay coefficients of variation were 6.5% and 8.7%, respectively. Melatonin RIA was performed according to Rollag and Niswender[13], using Stock Grand anti-melatonin antibody (Stock Grand, Surrey, UK). The recovery, accuracy and sensitivity for the melatonin RIA were 92%, 0.987 and 10 pg/mL, respectively. Intra- and inter-assay variations of melatonin were 9.0% and 15%, respectively.

2.5 Statistical Analysis

The data were presented as the means ± S.E. (M±S.E.). The data obtained from the weight of all collected organs and hormonal assay were Analyzed by One-Way Analysis of Variance (ANOVA[14]) followed by Student's-t test. Differences of mean were considered significant when P<0.05.

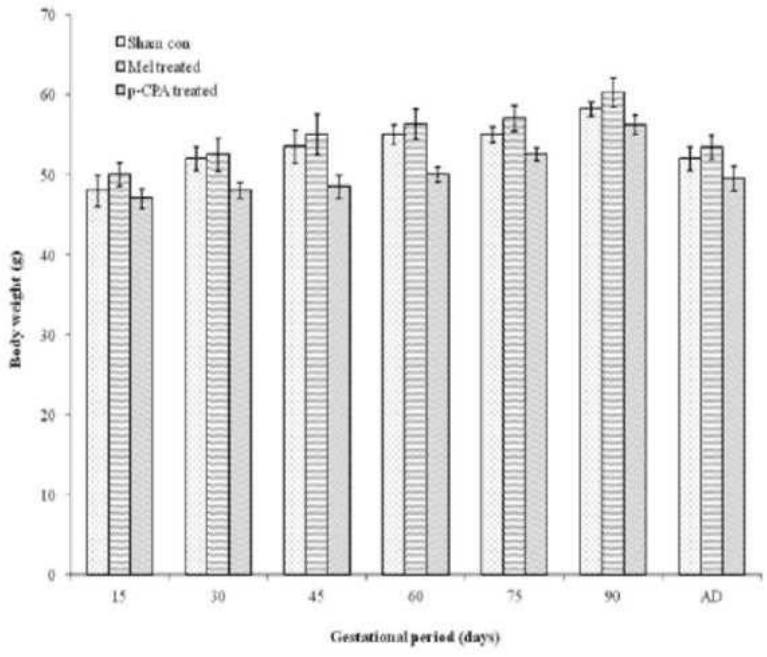

Figure 1. Histogram showing the effect of melatonin and pCPA on the body weight during gestation period of female Indian short-nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel / pCPA.

3. Results

3.1 Body Weight

Body weight of the pregnant female bats of each group increased throughout the gestational period. Around 100th day most of the pregnant bats delivered the litter and, therefore, a decrease in body weight was noted, which was then equivalent to the body weight of the early pregnant bats of 15th day. p-CPA treatment showed a decrease in body weight compared to sham control and melatonin-treated groups which might be due to the slow growth rate of the embryo as no abortion was noted (Figure 1).

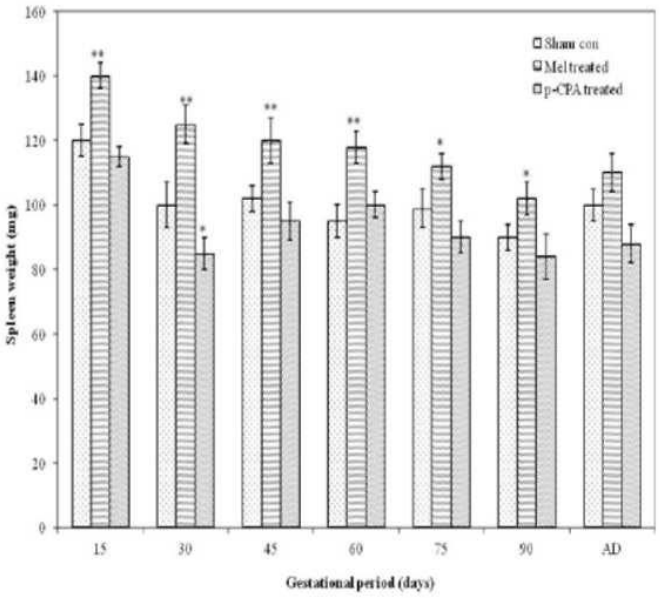

Figure 2. Histogram showing effect of melatonin and pCPA on weight of spleen during gestation period of female Indian short-nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel / pCPA, **P<0.01 Sham control vs Mel / pCPA.

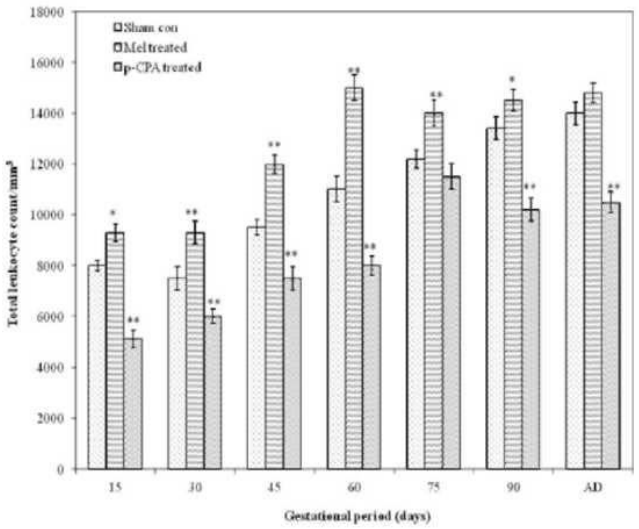

Figure 3. Histogram showing effect of melatonin and pCPA on total leukocyte count (%TLC) during gestation period of female Indian short-nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel / pCPA.**P<0.01 Sham control vs Mel / pCPA.

3.2 Spleen Weight

Spleen showed a decreasing trend in weight with increasing days of pregnancy. p-CPA injection reduced spleen weights of pregnant female bats only on 30th day of pregnancy and thereafter it had similar weight as control till delivery. Spleen weight of melatonin treated group was significantly high from 15th day onwards but maintained a decreasing trend till delivery as observed in sham control and p-CPA treated group (Figure 2).

3.3 Total Leukocyte Count and % Lymphocyte Count

Total leukocyte count in sham control increased from 45th day onwards and remained high even up to delivery.

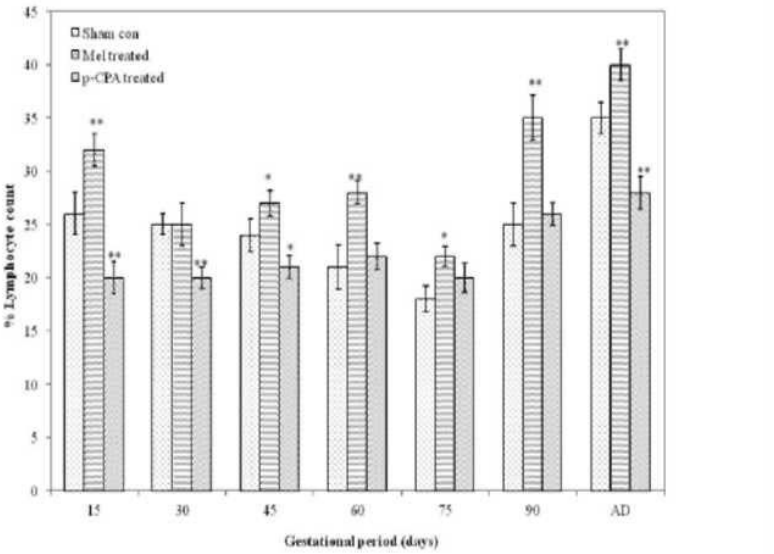

Figure 4. Histogram showing effect of melatonin and pCPA on percent lymphocyte count (%LC) during gestation period of female Indian short nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel and pCPA. **P<0.01 Sham control vs Mel and pCPA.

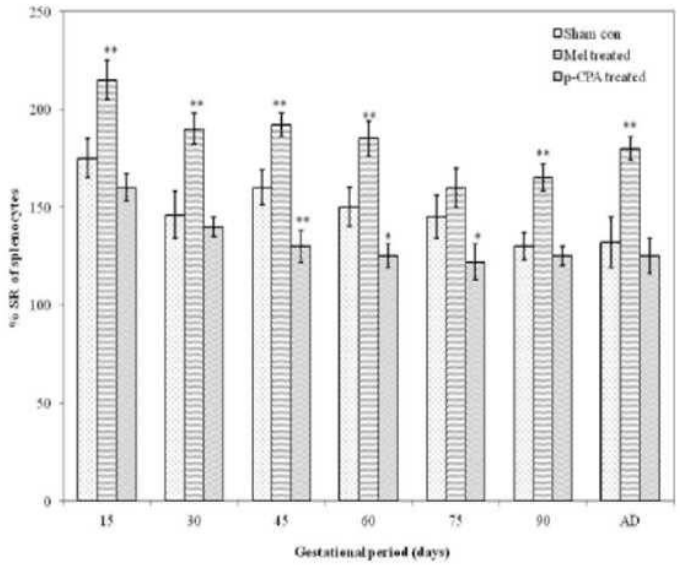

Figure 5. Histogram showing effect of melatonin and pCPA on percent stimulation ratio (%SR) of splenocytes during gestation period of female Indian short nosed fruit bat, *Cynopteryx sphinx.* Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel and pCPA. **P<0.01 Sham control vs Mel and pCPA.

Even after delivery this high TLC number was retained in sham control group. Total number of leukocytes of melatonin treated group steadily increased up to 60th day and remained high till delivery and even after delivery. p-CPA treated group showed significantly less total leukocyte count when compared with both sham control and melatonin treated groups.

In the case of percent lymphocyte count there was a significant decrease in p-CPA treated group from 15th day to 90th day of pregnancy and then it was high till delivery and even after delivery. In melatonin treated group lymphocyte count was significantly high (P<0.001) when compared with sham control and p-CPA treated groups and followed same pattern as TLC (Figure 3, 4).

3.4 Percent Stimulation Ratio (%SR)

Percent stimulation ratio decreased, following p-CPA treatment. It was significant from 45th to 75th day of pregnancy and remained low till delivery and even after delivery. In melatonin-treated group percent stimulation ratio was significantly high (P<0.001) from day 15 till after delivery when compared with sham control and p-CPA treated group (Figure 5).

3.5 Hormone Analysis

3.5.1 Melatonin

The night time melatonin level of plasma of pregnant female bats decreased significantly on 30th day in p-CPA treated group while it increased from 45th to 60th day of pregnancy in melatonin treated group. p-CPA treated group showed a steady decline in melatonin level from 30th day of pregnancy and maintained till delivery. In sham control group melatonin level showed a steady increase from 30th day to 100th day and decreased just before delivery. After delivery circulating melatonin level of sham control group as well as melatonin and p-CPA treated group showed lower concentration in plasma than on 15th day of pregnancy. Throughout the experimental period, the plasma level of melatonin in treated group was higher than that in sham control group while p-CPA treated group showed lower concentration (Figure 8).

3.5.2 Estradiol

Plasma estradiol level of sham control pregnant female bats gradually increased till delivery and decreased after delivery. p-CPA treated group presented a significant

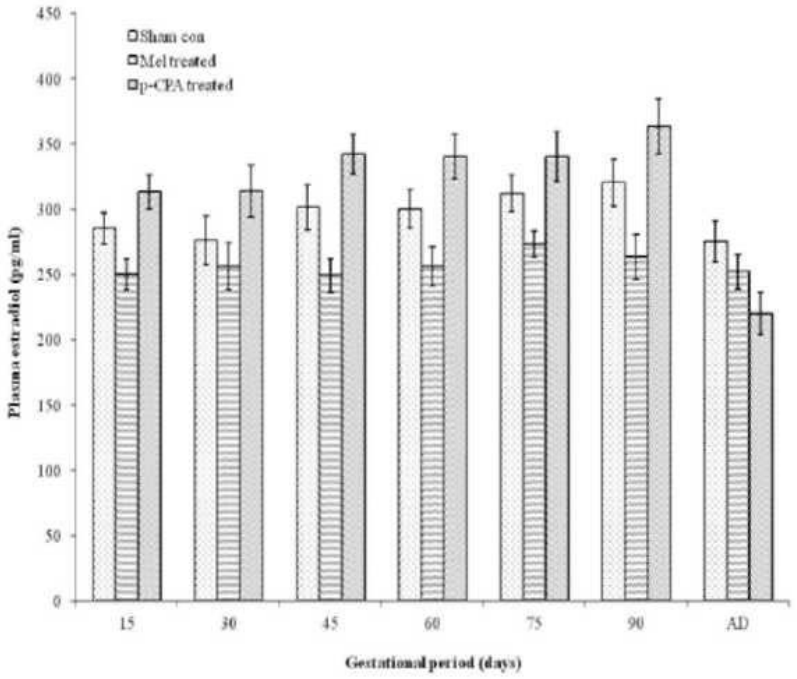

Figure 6. Histogram showing effect of melatonin and pCPA on the plasma estradiol level during gestation period of female Indian short nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel and pCPA. **P<0.01 Sham control vs Mel and pCPA.

increase in estradiol level compared to sham control groups. Melatonin treatment decreased estradiol level on all the days of pregnancy when compared with sham control and p-CPA treated groups (Figure 6).

3.5.3 Progesterone

Plasma level of progesterone increased from 45th day onwards in sham control and p-CPA treated groups and decreased in melatonin treated group up to 60th day of

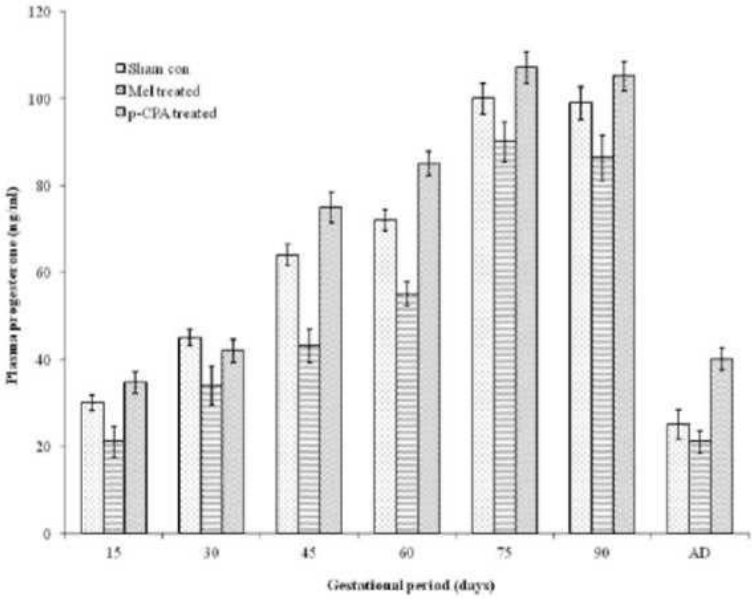

Figure 7. Histogram showing effect of melatonin and pCPA on the plasma progesterone level during gestation period of female Indian short nosed fruit bat, *Cynopteryx sphinx*. Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel and pCPA,**P<0.01 Sham control vs Mel and pCPA.

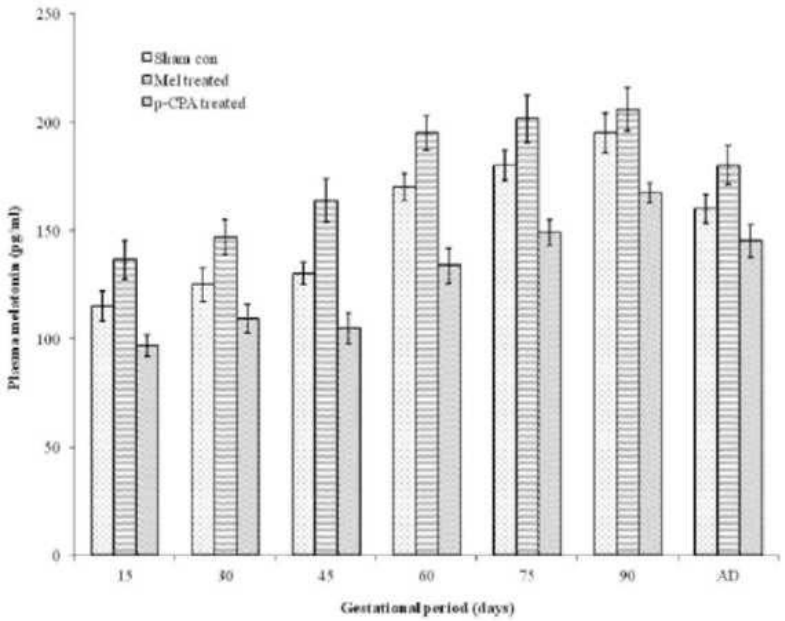

Figure 8. Histogram showing effect of melatonin and pCPA on the plasma melatonin level during gestation period of female Indian short nosed fruit bat, *Cynopteryx sphinx.* Vertical bar on each point represents mean + SE of 5 animals. *P<0.05 Sham control vs Mel and pCPA,**P<0.01 Sham control vs Mel and pCPA.

pregnancy and maintained this level till delivery. After delivery a sudden decline in progesterone level was noted in sham control and melatonin treated groups while this level was significantly high in p-CPA treated group (Figure 7).

4. Discussion

The ability of mothers to transmit antibodies to their offspring was established in mammals over 100 years ago[15]. In mammals, antibodies are transferred across the placenta prior to birth and through the colostrums and breast milk post-natally[16]. The amount and types of maternal antibodies transmitted may partially determine the survival probability of offspring[17]. Beyond guiding the diversity of the immune repertoire of offspring, maternal antibodies may also improve the strength of the offspring's immune response[18]. Stirrat[19] suggested that immune system may be modulated during pregnancy as part of a series of maternal adaptations necessary for successful fetal development.

This study was performed to record the immune status during pregnancy and the effect of melatonin and its indirect antagonist p-CPA on immunomodulation during pregnancy of the Indian short nosed fruit bat C. sphinx. We observed increase in body weight during gestational period in pregnant female bats in sham control, melatonin- and p-CPA-treated groups. This was mainly due to fat deposition in the lower abdominal area and fetal growth. The fat deposition was extra in melatonin treated pregnant females. The spleen weight showed decreasing trend during gestational period but in melatonin treated group decreased trend was high in comparison to other group i.e., sham control and p-CPA treated pregnant females.

Further, we found low hematological parameters in p-CPA treated pregnant female peripheral blood when compared with sham control and melatonin treated groups. In human pregnancy, a substantial increase in blood granulocyte and monocyte numbers is generally observed from the first trimester onwards[3,4], while important aspects of T-cell mediated responses become suppressed[20]. Little is known about pregnancy-associated alterations in blood cellular immunity in other mammalian species[21,22]. High number of total leukocyte was noted after 45th day of pregnancy in melatonin treated group, while percent lymphocyte count was low up to 75th day of pregnancy and there onwards high till delivery. Percent stimulation ratio was noted less in p-CPA treated groups with increasing gestation period. There was decreased lymphocyte responsiveness to mitogenic stimulation by Con-A during pregnancy in the human[23].

The estradiol level in melatonin treated pregnant female bats decreased when recorded on different gestational periods and when compared with the sham control and p-CPA treated groups. It might be due to reduced gonadotropin level in view of melatonin treatment. p-CPA treated bats presented a non-significant increase in estradiol level along with sham control group as noted on different days of gestational period. Plasma level of progesterone increased from 30th day to 75th day of pregnancy in pregnant female bats and maintained the level before delivery in sham control. Progesterone level was low on 15th day following melatonin treatment while it started increasing along with the level of progesterone noted in sham control and p-CPA treated groups upto 30th day. There was sudden decrease of progesterone level from 5th to 60th day. Further increase in progesterone level was noted in melatonin treated group, equivalent to progesterone level of sham control and p-CPA treated groups. It appears that effects of melatonin treatment at earlier days of pregnancy are different from later pregnancy. In later stage the progesterone level is maintained, while it decreases earlier. p-CPA treatment increased the progesterone level and helped in maintenance of pregnancy as noted in sham control group. Our results indicate that melatonin has an inverse relation with progesterone level during earlier embryonic developmental stage. Our finding draws support from the data of p-CPA treatment where suppression of melatonin increased progesterone level. Progesterone level was significantly low in all the groups after delivery. The immune function is up-regulated during the follicular phase because of higher concentration of estrogens while immune function decreased during the luteal phase, when estrogens were decreased and progesterone concentration was high[24]. Even though ovarian estrogen can up-regulate uterine and systemic immune functions in sheep, the suppressive effects of progesterone may override the effects of estrogens. The cyclic changes in progesterone concentrations are the major determinant of uterine immune function and the reduced progesterone concentrations permit estrogens to up-regulate uterine immune function. Melatonin level was high in melatonin treated group during the entire gestational period covered except after delivery. Exogenous administration of melatonin might increase the endogenous melatonin level in melatonin treated group while p-CPA,

acting as indirect antagonist of melatonin, reduced the melatonin level compared to sham control and melatonin treated groups. Communication between pineal gland and immune system is bidirectional, since circulating messages from activated immune cells (e.g., cytokines), inflammatory mediators and hormones reciprocally act on the pineal gland. Melatonin functions as an immunoregulatory factor in the development and maturation of the immune system and in progression of the immune response. It is now well established that amongst its numerous actions, melatonin is an immunomodulator, regulating the development, differentiation, and function of lymphoid tissues[25-27]. Indeed, diurnal and seasonal changes in immune function are thought to directly reflect changes in pineal melatonin production[28-30]. But, all immunological parameters were high in melatonin treated group when compared with sham control and p-CPA treated groups. Thus, we may suggest that immune status of pregnant bats is enhanced by exogenous melatonin treatment. Melatonin has been demonstrated to enhance both cell-mediated and humoral immune function in many species[31,32,10]. Melatonin treatment of both normal and immunocompromised house mice increased in vitro and in vivo antibody responses and T- helper cell activity[33,10]. Melatonin administration appears to stimulate humoral immunity by inhibiting apoptosis during early B cell development in mouse bone marrow[34]. Mounting evidences indicate that melatonin acts directly on immune tissues to modulate immune function. High affinity melatonin receptors have been localized on circulating lymphocytes from rodents, chicken and humans[35,36] and splenocytes in humans and a number of rodents[37-41].

Overall data analyses showed that immune status of pregnant bats is low following p-CPA treatment during pregnancy. p-CPA is indirect antagonist of melatonin, which inhibites melatonin synthesis thereby influencing the immune status of pregnant females.

Animals maintain the highest level of immune function that is energetically possible[42]. The observation that immune function is generally compromised during specific energetically demanding times such as winter breeding is consistent with the hypothesis that immune function is optimized[43]. Pregnant mammals also display compromised immune function among females[44]. The functional explanation for immune-suppression during pregnancy has been to protect the fetus from being attacked as foreign tissue by the maternal immune system[44].

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