MELATONIN MODULATES SPLENOCYTE IMMUNE RESPONSE IN THE FRESHWATER SNAKE, *NATRIX PISCATOR*

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Abstract.—**The role of melatonin in altering cell mediated nonspecific immune responses has been documented in mammals, but not in reptiles. We designed our study to evaluate the role of melatonin in altering innate immune responses of splenocytes in the freshwater snake, *Natrix piscator*. We gave the melatonin injections (dose: 5 μg/g body weight and 10 μg/g body weight) during evening hours. Control animals received saline injection. We sacrificed the 5 μg/g group of snakes after 10 d, and the 10 μg/g group after 20 d. We studied alteration in lymphoid organ mass, splenic macrophage phagocytosis, nitric oxide (NO) production (Nitrite assay), superoxide production (NBT reduction assay), and lymphoproliferation (MTT assay). We did not observe any consistent and significant change in phagocytic response of splenic macrophages harvested from snakes treated with either dose of melatonin for different duration. Nitrite release and superoxide production by splenic macrophages were significantly higher in snakes receiving melatonin injections. We found that exogenous melatonin enhanced the mitogen-induced splenic lymphocyte proliferation only at the dose 10 μg/g body weight, but not at that of 5 μg. *In vitro* melatonin also enhanced the mitogen-induced splenic lymphocyte proliferation, and we observed vigorous response in splenocytes harvested from snakes receiving melatonin 10 μg/g body weight for 20 d, when splenocytes were induced by 10 μg/mL of both Con A and PHA and by 20 μg/mL of LPS.**

Key Words.—immune response; macrophage; melatonin; *Natrix piscator;* proliferation

INTRODUCTION

Although much is known about the functioning of the immune system in mammals, relatively less is known for non-mammalian vertebrates. Reptiles are ectothermic amniotes, providing the key link between ectothermic anamniotic fishes and amphibians, and endothermic amniotic birds and mammals; a greater understanding of reptilian immunity will provide important insights into the evolutionary history of vertebrate immunity (Zimmerman et al. 2010). Earlier studies have demonstrated that reptiles are endowed with an advanced type of immune system; bone marrow, thymus, spleen, and gut-associated lymphoid tissues are well developed (Bockmann 1970; Coe 1972; Hussein et al. 1979; Mansour et al. 1980). The functional relevance of the reptilian spleen has been demonstrated, as splenoctomy in lizards causes an acute inhibition of several immune responses, and the spleen is the major site of immune responses to blood-borne antigens and is also a site of hematopoiesis (Batista and Harwood 2009).

Several studies have examined sex differences in lymphocyte proliferation in reptiles but the results are contradictory. There were no significant differences in the proliferation between males and females in response to Con A (Concanavalin A) and PHA (phytohemaglutinin) in the Caspian Pond Turtle, *Mauremys caspica* (Munoz and Fuente 2001). Likewise, there was no effect of sex or plasma testosterone concentration on lymphocyte proliferation from Loggerhead Sea Turtles (*Caretta caretta*) in response to Con A and PHA (Keller et al. 2005).

However, sex differences were detected in the proliferation of lymphocytes from the Striped Sand Snake (*Psammophis sibilans*), with females having higher proliferation in response to Con A and PHA than males (Saad 1989). Sexual dimorphism and possible role of sex steroids in innate immune response have also been reported in lizards (Mondal and Rai 1999). Sex steroids are not the only hormones that regulate immune functions. Melatonin (N acetyl 5-Methoxytryptamine) is shown to regulate immune functions in mammals. Hence, it is of interest to explore the role of melatonin in altering immune responses in reptiles.

Farag and El Ridi (1986) found that lymphocytes from the Striped Sand Snake (*Psammophis sibilans*) had the highest proliferation in a mixed leukocyte reaction during the spring and autumn. Proliferation of lymphocytes from the Caspian Pond Turtle in response to Con A and PHA was strongest in spring but was significantly diminished in summer, autumn, and winter (Munoz and Fuente 2001). Melatonin concentration peaks during the night and bottoms during day in almost every species examined. Thus environmental cue, somehow, are translated into internal melatonin rhythm and seasonal fluctuations in melatonin concentrations are associated with disease prevalence and immune functions. It has been established that melatonin, primarily produced by pineal gland, is an immunomodulator regulating the development, differentiation and function of lymphoid tissues and its component cells, and a good deal of information is available regarding effect of melatonin on immune responses in mammals and birds

(Hotchkiss and Nelson 2002; Skwarlo-Sonta 2002; Miller et al. 2006). The results from various studies indicate that melatonin has immunostimulatory effects in mammals. There are known differences between mammals and birds related to immunomodulatory effect of melatonin exerted *in vivo* (Markowska et al. 2001, 2002). Lack of immunostimulatory effect in birds is suggested due to difference in basic immunology between species. For example, thymic hormones differ in mammalian versus avian species; and additional evidences suggest that activation of opioid system in avian species is not immunostimulatory, as it is in mammalian species (Skwarlo-Sonta 2002).

Attempts to understand mammalian immune system will be aided by a more systematic approach to investigating immune function across vertebrate groups. Reptiles are a crucial phylogenic group from which both birds and mammals evolved. Also, reptiles are the only ectothermic amniotes, and therefore become a pivotal group to study to provide significant insights into both the evolution and functioning of the immune system. Reptiles are generally long-lived, with an extended period of growth and maturation early in life. However, reptiles are unable to internally regulate their body temperature, and undergo strong seasonal change in behavior associated with environmental temperatures (Zimmerman et al. 2010). Collectively, these characteristics may have profound effects on how reptiles partition energy resources to self-maintenance activities, including immune function. Despite similarities to mammals in the generation of antibody diversity, reptile humoral responses are slower. After immunization, antibodies can be detected after a latent period of around one week but often do not peak until six or eight weeks post-immunization (Work et al. 2000). Although biologists have been investigating the reptilian immune system for some time, there is still much to learn. Additional integrative studies are required to understand the complex interaction among the endocrine and immune system in regulation of immune function in more non-mammalian species. Therefore, we conducted our study to examine cell-mediated immune responses in relation to melatonin treatment in an ophidian species, the freshwater snake, *Natrix piscator*.

MATERIALS AND METHODS

Study animal.—We obtained the snakes, which weighed 80–120 g, from a local supplier who collected the animals in the suburbs of Varanasi (28°18'N; 83°1'E) from February to March, 2011(max. temp. 27–32 °C, min. temp. 14–18 °C; 11–12 hours of sunlight; relative humidity 55–68%), the reproductively inactive period (Haldar and Pandey 1989). We housed the animals in wood and wire net cages (size 50 × 30 × 30 cm) in the institutional animal room at 24 ± 2°C. Each cage had an earthen bowl (4 L capacity) filled with water, which accommodated 4–5 snakes. We fed the snakes with small fish (*Puntius puntio*) once weekly. We followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Ministry of Statistics & Programme Implementation, Government of India, in maintenance and sacrifice of animals.

Experimental procedure.—Captivity lasted two weeks to relieve the capture-related stress and to acclimate the animals to the laboratory conditions. Following acclimation, we divided the snakes into six groups: four experimental and two control groups (each group contained five animals). To animals in the experimental groups we gave an intraperitonial injection of melatonin (5 and 10 µg/g body mass) daily and sacrificed them on the day following the 10th and 20th injection, respectively. We prepared the stock solution of melatonin by dissolving it in few drops of ethyl alcohol then diluted with distilled water. To animals in the control groups, we gave intraperitonial injection of vehicle saline (a few drops of ethyl alcohol in distilled water) daily and also sacrificed them on the day following the 10th and 20th injection, respectively. We chose the dose of melatonin injection based on earlier studies (Hriscu 2004; Cuesta et al. 2007) and gave all injections between 1600 and 1700. On the day after expiry of treatment, we sacrificed the animals under mild anaesthesia of ether. We weighed the spleen, thymus, gonads, and gonoducts in mg and calculated their relative mass. Soon after, we used the spleens to study the immune parameters.

Preparation of macrophage monolayer.—Under aseptic conditions, we macerated the excised spleen through a nylon strainer of pore size < 100 µm into complete culture medium (2 mL/spleen) to get single cell suspension under a sterile laminar flow hood. We checked the cell viability using a trypan blue exclusion test, which exceeded 95%, and determined the spleen cellularity using a hemocytometer. We followed the method of Mondal and Rai (1999) for splenic macrophage monolayer preparation and phagocytic assay. Briefly, we flooded the splenic cell suspension (200 µL) onto washed and sterilized glass slides, and allowed the macrophages to adhere by incubating them at 25 °C in humidified CO_2 atmosphere for 90 min. Non adherent cells were washed off with 0.2 M phosphate buffer saline (PBS; pH 7.2). We prepared the macrophage monolayer in duplicate from each spleen. In the adherent cell population, more than 90% of the cells were macrophages as judged by their morphology.

Assays.—For the phagocytic assay, we used the yeast cells as target. We prepared the yeast cell suspension by mixing 20 mg of commercial baker's yeast (*Saccharomyces cerevisae*) into 10 mL of 0.2 M PBS. We kept the suspension at 80 °C for 15 min, then washed the cells three times in PBS and finally

suspended in complete culture medium to get a concentration of 1×10^8 cells/mL. We flooded the splenic macrophage monolayer, prepared as above, with yeast cell suspension, and allowed phagocytosis to proceed by incubation for 90 min at 25 °C in humidified CO_2 atmosphere. We rinsed the slides in PBS, fixed in methanol, stained with Giemsa, and examined under oil immersion. For each slide, we examined 100 macrophages randomly without any predetermined sequence. We determined the phagocytic index by calculating the average number of yeast cells engulfed by a single macrophage.

We performed Nitro Blue Tetrazolium (NBT) assay following methods of Berger and Slapnickova (2003). We counted and adjusted the spleen cells to 2×10^6 cells/mL in complete culture medium RPMI 1640 (developed at Roswell Park Memorial Institute; Himedia Pvt. Ltd., Mumbai, India). We mixed spleen cell suspension (50 µL having 1×10^5 cells) with 50 µL of RPMI containing NBT (1 mg/mL) in 96 well culture plate in triplicates from each spleen. One well with culture medium alone served as blank. We then incubated the plates in CO_2 atmosphere at 25 °C for 2 h, centrifuged at $700 \times g$, washed with PBS, fixed in 70% methanol and mixed 20 µL of 0.1% triton X-100 in each well. We dissolved the formazan crystals by mixing 120 µL KOH (2 M) and 140 µL DMSO in each well. We measured optical density at 620 nm using an ELISA reader (Thermo Multiscan; Waltham, Massachusetts, USA).

Nitric oxide (NO), effector molecule of macrophage cytotoxicity, is a highly unstable compound produced from L-arginine by enzyme Nitric oxide synthase (NOS). Soon after production, NO decomposes to other nitrogen oxides, such as nitrate (NO_2^-) and nitrite (NO_3^-), known as Reactive Nitrogen Intermediate (RNI). We measured nitrite content by the method of Ding et al. (1988). Briefly, we added 100 µL of splenocytes (1×10^6 cells/mL) in each well of a 96 well culture plate. After 2 h of incubation at 25 °C, we washed cells with PBS. We added 100 µL of fresh culture medium to each well, and incubated the plates in CO_2 atmosphere at 25 °C for 24 h. We centrifuged the plates at $200 \times g$ to collect supernatant. We then mixed equal volume of supernatant and Griess reagent (1% sulfanilamide in 3 N HCl and 0.1% naphthylenediamine dihydrochloride in distilled water), and measured optical density of the solution at 540 nm with the ELISA reader. Culture medium alone served as a blank. We took triplicate cultures from spleen of each experimental animal.

We treated splenic single cell suspension with hemolysate buffer, washed with 0.2 M PBS (pH 7.2) twice and resuspended in complete culture medium. We isolated the lymphocytes by density gradient centrifugation at $400 \times g$ for 30 min at 8 °C using lymphocyte separation medium (HiSep, Density 1.077 g/mL). We washed the isolated lymphocytes three times with PBS, counted and assessed for viability using the trypan blue exclusion test. We adjusted the

viable cells (> 95%) to 2×10^6 cells/mL in culture medium.

We assessed the lymphocyte proliferation using colorimetric assay based on tetrazolium salt (MTT) following the methods of Berridge et al. (2005). Tetrazolium salts are reduced into a colored formazan product in mitochondria of metabolically active cells. The quantity of formazan product as measured by amount of absorbance at 570 nm light is directly proportional to the number of living cells in culture (Cory et al. 1991). We made stock solution (1 mg/mL) of mitogens in 0.2 M PBS and further diluted with culture medium.

We seeded the splenic lymphocytes (1×10^5 cells) in 50 µL of medium/well in a flat-bottom 96 well culture plate. We then added the mitogens (50 µL): Con A at final concentration of 5, 10 and 20 µg/mL; LPS of 10 and 20 µg/mL; PHA of 5 and 10 µg/mL. To make the final volume 200 µL/well, we added culture medium. Cell suspensions (50 µL) with 150 µL of mitogen-free culture medium in well represented basal or spontaneous proliferation. Additional well containing 200 µL of culture medium served as blank. Following incubation in humidified CO_2 atmosphere at 25 °C for 48 h, we added 20 µL of MTT reagent (5 mg/mL) to each well, and again incubated the plates. After incubation, we aspirated the supernatant, and dissolved the reaction product blue formazan in 100 µL of DMSO. We measured the absorbance at 570 nm in ELISA plate reader. Following blank substraction, we averaged the triplicates. We calculated the stimulation index (SI) as SI = OD of stimulated culture / OD of unstimulated culture.

Effect of in vitro melatonin on proliferation.—We seeded the splenic lymphocytes (50 µL) from *in vivo* melatonin treated snakes with mitogens Con A, Lipopolysaccharide (LPS) and Phytohemaglutinin (PHA; 50 µL), as described above. We added different concentrations (100, 200 and 500 pg/mL; final concentration) of melatonin (50 µL) and 50 µL of culture medium to the cultures to make final volume 200 µL. We selected the concentrations of melatonin based on earlier studies (Kliger et al. 2000; Prendergast and Nelson 2001; Prendergast et al. 2002). We processed the culture plates as described above. Because prior exposure may affect the cellular response to *in vitro* exposure, we handled the data to reduce variability, and presented the cellular proliferative response to *in vitro* melatonin as percentage cell proliferation. We prepared cultures with no melatonin and no mitogen to provide basal proliferation. Control cultures contained lymphocytes and mitogens; while treatment culture contained lymphocytes, mitogens, and *in vitro* melatonin. We substracted the OD of basal cultures (no *in vitro* melatonin and no mitogen) from treatment OD (with mitogen and *in vitro* melatonin) and also from control OD (with mitogen and no *in vitro* melatonin). Then, we considered OD of control cultures to be the

baseline and assigned a value of 100%. Percentage cell proliferation of *in vitro* melatonin treated cultures was calculated relative to proliferation in control cultures: Percentage cell proliferation = {(treatment OD - control OD) / control OD} x 100 + 100.

Statistical analysis.—We have presented the data as mean ± SE. We analyzed the data for gonad, gonoduct, thymys, and spleen weight and spleen cellularity using Student's *t*-tests and used ANOVA followed by Post-hoc Student-Newman-Keul's multiple range test for the data of phagocytosis, NBT, nitrite, and splenocyte proliferation. For all tests, $\alpha \leq 0.05$.

RESULTS

Effect of in vivo melatonin.—Administration of melatonin in both doses (5 and 10 μg/g body weight) in snakes of experimental groups caused a significant decrease in mass of gonad ($t = 4.87$, df = 3, $P < 0.05$) and gonoduct ($t = 3.93$, df = 3, $P < 0.05$), when compared to vehicle treated control ones. The degree of reduction was the same for gonoducts at both doses at 10 d ($t = 3.93$, df = 3, $P = 0.01$ and $t = 3.25$, df = 3, $P = 0.02$, respectively) and 20 d ($t = 3.50$, df = 3, $P = 0.009$ and $t = 3.58$, df = 3, $P = 0.008$, respectively); while that in the gonad was more pronounced at 20 d in animals injected with melatonin 10 μg/g body weight ($t = 5.63$, df = 3, $P = 0.005$). Though the average spleen mass was slightly enhanced in animals receiving melatonin 10 μg/g body, there was no significant difference in spleen mass and its cellularity in melatonin injected animals, either in relation to dose or duration of treatment ($t = 1.08$, df = 5, $P > 0.05$). We found increased thymic mass when snakes were treated with *in vivo* melatonin. Increase in thymic mass was dependent on the dose and duration of melatonin treatment. We obtained maximal increase in thymic mass in snakes treated with 10 μg/g body weight melatonin for 10 d. Prolonged duration (20 d) of melatonin treatment also increased the thymus mass, but this effect was less pronounced, when compared to 10 d treatment (Table 1).

We did not find any consistent and significant change in phagocytic response of splenic macrophages
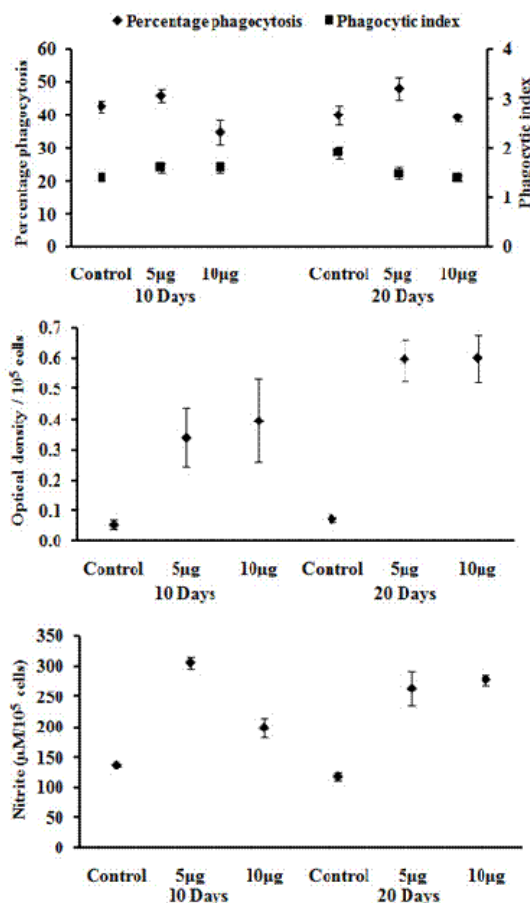
FIGURE. 1. Effect of *in vivo* melatonin (5 μg and 10 μg/g body weight) on splenic macrophage phagocytosis (upper panel), NBT reduction (middle panel), and nitrite release (lower panel) by splenocytes in *Natrix piscator*.

harvested from snakes treated with either dose of melatonin for different duration, when compared to that of respective controls. Super oxide production, as judged by NBT reduction test, significantly ($F_{5,30} = 8.797$, $P < 0.0001$) increased in the cultures obtained from animals treated with melatonin. We also found that *in vivo* melatonin, either dose, for 10 and 20 d had stimulatory effect on NBT reduction. Maximal increase in NBT reduction occurred in splenocytes

TABLE 1. Effect of exogenous administration of melatonin (5 and 10 μg/100g body weight) to *Natrix piscator*. Values with * indicate significant difference ($P < 0.05$) when compared to values in control animals.

| Relative organ weight	10 days			20 days		
	Control	5 μg	10 μg	Control	5 μg	10 μg
Gonad (g/100g body weight)	0.51 ± 0.05	0.25 ± 0.01*	0.28 ± 0.03*	0.59 ± 0.06	0.34 ± 0.04*	0.21 ± 0.01*
Gonoduct (g/100g body weight)	0.92 ± 0.12	0.42 ± 0.02*	0.51 ± 0.02*	0.92 ± 0.12	0.42 ± 0.07*	0.41 ± 0.07*
Thymus (mg/100mg body weight)	8.23 ± 2.13	12.2 ± 1.96	18.6 ± 2.97*	8.23 ± 2.13	9.67 ± 1.62	13.1 ± 0.82
Spleen (mg/100mg body weight)	38.8 ± 3.88	50.0 ± 10.11	62.1 ± 13.21	42.4 ± 3.33	42.9 ± 7.18	53.2 ± 8.26
Spleen cellularity (×10⁶/mg spleen)	1.29 ± 0.25	1.60 ± 0.29	1.15 ± 0.31	1.08 ± 0.12	1.36 ± 0.11	0.76 ± 0.19
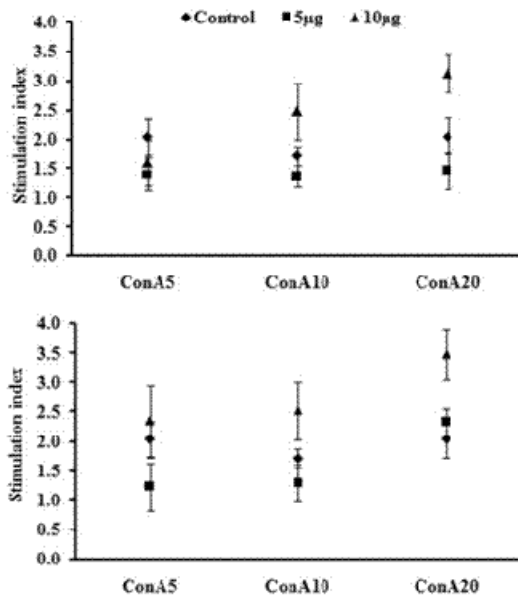
FIGURE. 2. Effect of *in vivo* melatonin (5 µg and 10 µg/g body weight) for 10 d (upper panel) and 20 d (lower panel) on Con A-induced splenic lymphocyte proliferation in *Natrix piscator*. ConA 5, ConA 10, and ConA 20; Con A concentration 5, 10, and 20 µg/mL, respectively.

FIGURE. 4. Effect of *in vivo* melatonin (5 µg and 10 µg/g body weight) for 10 d (upper panel) and 20 d (lower panel) on LPS induced splenic lymphocyte proliferation in *Natrix piscator*. LPS 10 and LPS 20; LPS concentration 10 and 20 µg/mL, respectively.

culture obtained from animals treated with 10 µg/g body weight melatonin for 20 d. Nitrite release in cultures of splenocytes obtained from animals treated with melatonin was significantly ($F_{5,45} = 34.048$, $P < 0.001$) enhanced in comparison to that of respective controls. We also observed that 5 µg/g body weight melatonin had more pronounced effect than 10 µg/g
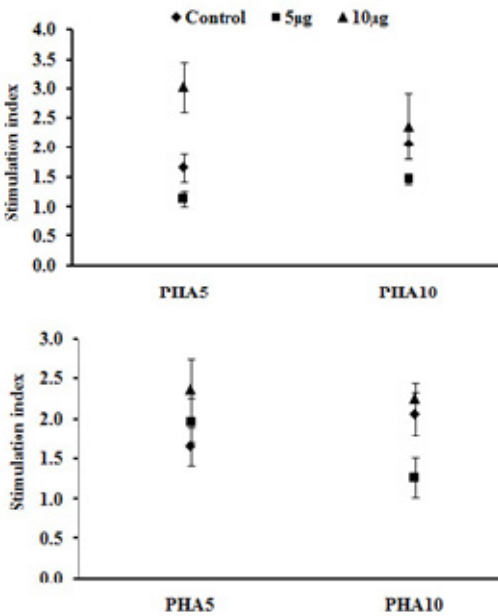

FIGURE. 3. Effect of *in vivo* melatonin (5 µg and 10 µg/g body weight) for 10 d (upper panel) and 20 d (lower panel) on PHA-induced splenic lymphocyte proliferation in *Natrix piscator*. PHA 5 and PHA 10; PHA concentration 5 and 10 µg/mL, respectively.
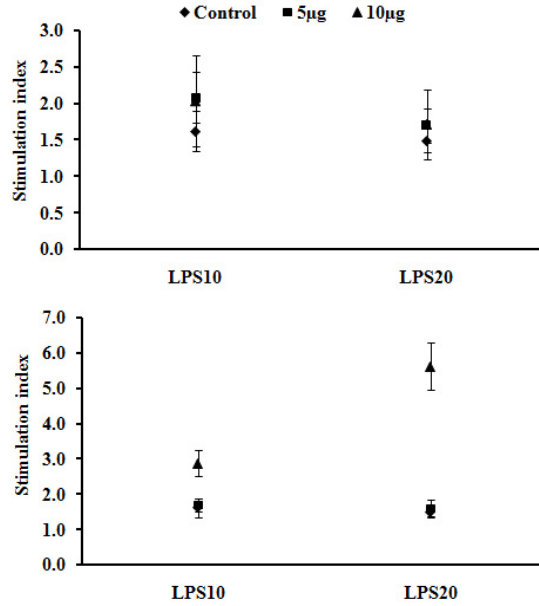
body weight melatonin treated for 10 d (Fig. 1). Melatonin administration (*in vivo*) did not affect the Con A-induced splenocyte proliferation in animals treated with 5 µg/g body melatonin either for 10 or 20 d. However, the average proliferative response of splenocytes obtained from animals injected with 10 µg/g body melatonin increased significantly ($F_{17,90} = 3.154$, $P < 0.001$) in a manner dependent on the concentration of Con A; that was more pronounced and significant when splenocytes were induced by 20 µg/mL Con A (Fig. 2). PHA-induced proliferative response of splenocytes obtained from animals injected with 10 µg/g body melatonin for 10 d was enhanced significantly ($F_{11,60} = 3.209$, $P = 0.002$) only at 5 µg/mL concentration of mitogen, as compared to respective control (Fig. 3). We did not find any effect of *in vivo* melatonin (5 µg) on LPS-induced splenocytes proliferative response either at 10 or 20 d treatment; but 10 µg melatonin injected for 20 d caused a significant ($F_{11,60} = 9.722$, $P < 0.001$) enhancement in proliferative response of LPS-induced splenocytes, and the response was more vigorous at 20 µg/mL mitogen concentration (Fig. 4).

Effect of in vitro melatonin.—There was no consistent proliferative response of splenocytes in relation to administered doses of *in vivo* melatonin. Therefore, when we have reported the overall effect of *in vitro* melatonin, we have combined the percentage cell proliferation for both doses of *in vivo* melatonin. Addition of melatonin (100, 200, and 500 pg/mL) to splenic lymphocyte culture *in vitro* had significant ($F_{7,84} = 7.895$, $P < 0.001$) stimulatory effect on
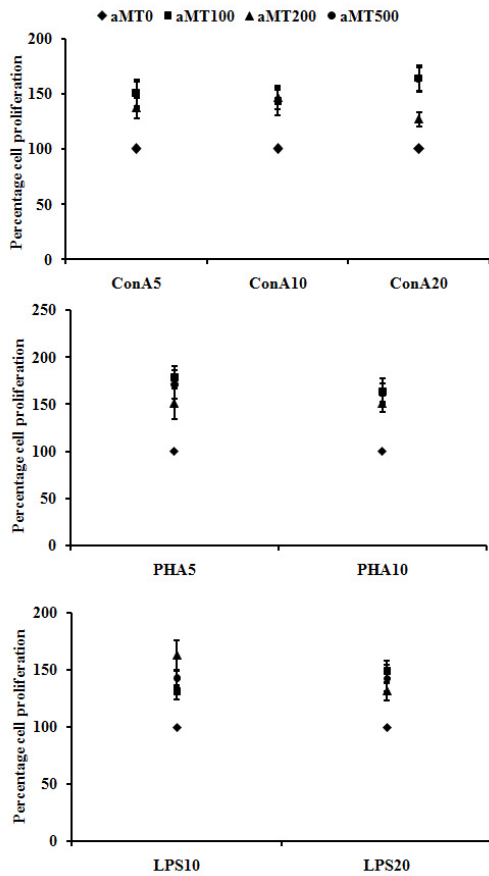
FIGURE. 5. Effect of *in vitro* melatonin (aMT0, aMT100, aMT200, and aMT500; melatonin concentration 0, 100, 200, and 500 pg/mL, respectively) on mitogen-induced splenic lymphocyte proliferation of *in vivo* melatonin-treated snake, *Natrix piscator*, for 10 d.
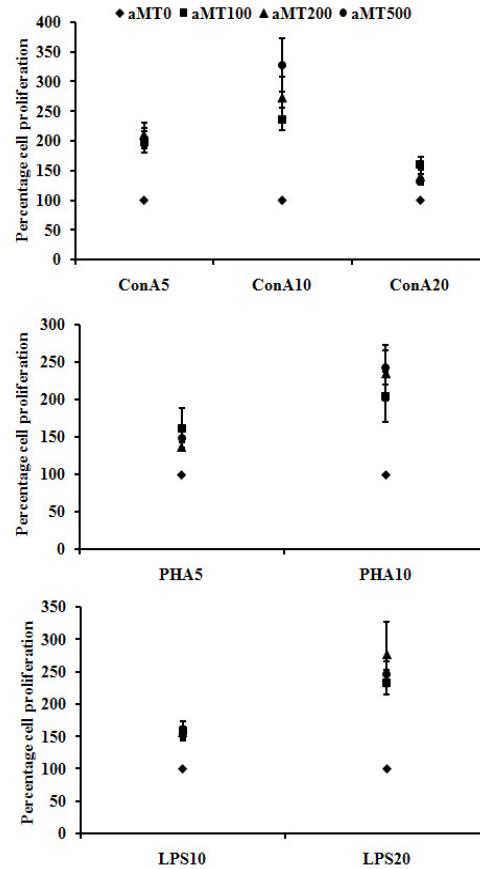
FIGURE. 6. Effect of *in vitro* melatonin (aMT0, aMT100, aMT200, and aMT500; melatonin concentration 0, 100, 200, and 500 pg/mL, respectively) on mitogen-induced splenic lymphocyte proliferation of *in vivo* melatonin-treated snake, *Natrix piscator*, for 20 d.

mitogen-induced proliferation of splenocytes obtained from snakes that were already treated with *in vivo* melatonin for 10 and 20 d. However, we found more stimulatory effect in 20 d splenocytes, when induced by 10 µg/mL of Con A and PHA and 20 µg/mL of LPS (Figs. 5 and 6).

DISCUSSION

In this study, the first of its kind, we found that melatonin enhances the immune function in the freshwater snake, *Natrix piscator*. Melatonin acts as antigonadal hormone regressing the size of reproductive organs significantly. Mitogen induced splenic lymphocyte proliferation was significantly increased in the snakes treated with *in vivo* doses of melatonin when compared to vehicle-treated controls. We did not find any significant change in splenic macrophage phagocytosis in treated animals when compared to control animals suggesting that certain critical levels of immunity are essential even in low concentrations of melatonin. Oxidative burst as measured by NBT assay and nitrite release was also increased significantly and increase was dependent on

the concentrations of the melatonin used.

In the only study of the immune response of a reptile, Singh (2008), who studied only the leucogram, thymus, and spleen mass in a lacertilian species, *Calotes versicolor* in relation to melatonin treatment, reported increase in thymus and spleen mass following melatonin administration. Otherwise, this is the first study of the effect of melatonin on cellular immune responses (Phagocytic, cytotoxic, and lymphoproliferative) in a reptile. Other studies have used endotherms to determine the role of melatonin. The antigonadal role of melatonin is well established in seasonally breeding mammals and birds (Chowdhury et al. 2008), and we found the same response in the ophidian species we studied: melatonin reduced the gonad and gonoduct mass significantly. Lymphoid organs are the important elements of non-specific immunity, and variations in lymphoid organs mass and leucocyte counts are widely taken into consideration to assess the immune status of the animals (Hotchkiss and Nelson 2002). In this study, spleen mass and cellularity increased, but not significantly; while we found a significant increase in thymus mass in melatonin treated snakes. Increase in

thymus and spleen mass following *in vivo* melatonin administration has been reported in mammals that received a pinealectomy, with a resultant elimination of circulating melatonin and a decrease in thymus and spleen mass (Haldar et al. 2004). In birds, there is no unanimity in the observations, as pinealectomy has resulted in the reduction in survival rate, retardation of development of thymus, spleen and bursa of fabricius, and depletion of lymphocytes in thymus, bursa, and spleen, and some studies suggest that exogenous melatonin treatment decreases both spleen and bursal mass (Giannessi et al. 1992). On the other hand, Singh and Haldar (2005), and Singh et al. (2006) have reported increase in weight of lymphoid organs in a tropical bird. Moore and Siopes (2002) have observed increase in bursal mass, but not in thymus and spleen following *in vivo* melatonin treatment in birds.

We failed to find any effect of melatonin on macrophage phagocytosis; while avian heterophil phagocytic function increases in a dose-dependent manner following *in vivo* and *in vitro* treatment of melatonin (Terron et al. 2003, 2004). On other hand, Roy et al. (2008) have reported the concentration-dependent inhibitory effect of *in vitro* melatonin on phagocytic activity of splenic macrophages in a freshwater teleost; while Cuesta et al. (2007) have reported diverse effects of melatonin on phagocytic immune response in fish, depending on the fish species. This suggests an acquisition of melatonin function in mammals where phagocytic activity is enhanced. There is a gradual shift from inhibitory action of melatonin on phagocytosis in fish to loss of this function in reptiles as melatonin caused no change in phagocytosis in our study. Furthermore, a new immunostimulatory function for melatonin (phagocytosis) has been found in mammals (Pawlak et al. 2005). This new function in mammals arose possibly because mammals can regulate their body temperature and they can maintain a critical level of immunity throughout year, which is essential for survival. During the periods of pathogen stress, this acquired function of melatonin helps them to combat diseases more effectively. However, reptiles show a seasonal cycle of temperature variation and to maintain a critical level of immunity, some immune functions, such as phagocytosis, are kept independent of melatonin concentration.

The reduction of NBT is carried out by the superoxide anion (O_2^-) produced by granulocytes and macrophages. The NBT reduction test is a measure of activation of oxidative burst, which has a high reactive microbicidal effect in response to antigenic stimulation (Jorens et al. 1995). In the absence of antigenic stimulation, the NBT reduction test is also an indirect measure of the intracellular hexosemonophosphate shunt activity (Park et al. 1968). In our study, we demonstrated that resting splenic macrophages seems to be able to produce significantly increased amounts of O_2^- in melatonin-injected snakes, maybe via stimulation of metabolic pathway. In human

neutrophils, melatonin resulted in an increase of the respiratory burst in response to PMA (Pieri et al. 1998); while oral administration of melatonin decreases NBT reduction in Old Ring Dove, *Streptopelia risoria* (Rodriguez et al. 1998).

The mitogen-induced lymphocyte proliferation assay is perhaps the most widely used functional assessment of the cellular arm of immune system. Proliferative response of peripheral blood lymphocytes stimulated by mitogens (Con A, PHA, LPS, Pokweed mitogen and Egg white lysozyme) has been reported in a few reptilian species (Cray et al. 2001; Munoz and Fuente 2003; Keller et al. 2005, 2006). In our study, *in vivo* melatonin has enhanced the mitogen-induced splenic lymphocyte proliferation only at the dose 10 µg/g body weight, but not at that of 5 µg. *In vitro* melatonin also invariably has enhanced the mitogen-induced splenic lymphocyte proliferation, and vigorous response has been observed in splenocytes harvested from snakes receiving 10 µg/g body weight melatonin for 20 d, when splenocytes were induced by T – and B – cell mitogens at different concentrations, viz. 10 µg/mL of Con A and PHA, and 20 µg/mL of LPS.

Treatment with exogenous melatonin (*in vivo*) has been reported to reverse the immune impairment caused by aging and ovariectomy (Baeza et al. 2009), to enhance the cell-mediated as well as humoral immune responses in mammals and birds (Haldar et al. 2004), and to increase the mitogen-induced lymphoproliferation (Kriegsfeld 2001; Rai et al. 2005; Singh et al. 2006). On the other hand, Santello et al. (2008) have reported suppressive action of *in vivo* melatonin on lymphoproliferation in mice infected with *Trypanosoma cruzi*. Thus, *in vivo* models to test immunomodulatory role of melatonin, in general, reveals immunoenhancing properties of the hormone. However, *in vitro* studies of a hormone on immune function seems valuable, for the confounding influence of concurrent circulating hormones as *in vivo*, are eliminated. Results of many *in vitro* melatonin studies are contradictory. Melatonin *in vitro* has been reported to enhance the mitogen-induced lymphoproliferation in mammals and birds (Drazen et al. 2000, 2001; Prendergast et al. 2002; Singh et al. 2006); while many authors have either claimed no effect on resting lymphocytes activated with mitogen, as melatonin at low or high concentrations failed to activate lymphocytes proliferation in birds and mammals, including humans (Konakchieva et al. 1995; Pahlavani and Harris 1997; Rogers et al. 1997), or claimed inhibitory effect (Konakchieva et al. 1995; Markowska et al. 2001, 2002). Further, the effect of melatonin on immune function may depend on the species, the age and sex of the individual, the physiological status of immune system, the parameters examined, the season, and the dose and route of melatonin administration. Taken together, the results of our study demonstrate that *in vivo* melatonin treatment enhances innate immunity, in general,

except phagocytosis, in *N. piscator*. In addition, enhanced lymphoproliferative response to *in vitro* melatonin supports the view that melatonin exerts a direct effect on splenocyte proliferation, and the differences in concentration of mitogens may have an effect on melatonin enhancement of mitogen-induced proliferation.

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