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DAY NIGHT VARIATION IN PHAGOCYTOSIS AND SUPEROXIDE PRODUCTION BY LEUCOCYTES IN FRESHWATER SNAKE, *NATRIX PISCATOR*

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ABSTRACT
Aim of the present study was to understand the diurnal variation in phagocytosis and superoxide production by blood leucocytes in the fresh water snake, *Natrix piscator*. Leucocyte phagocytosis and superoxide production are important constituents of innate immune response and form the first line of host defense. Yeast (*Saccharomyces cerevisae*) cells were used as target cell to study phagocytosis. Oxidative burst activity was measured by reduction of a tetrazolium dye. Snakes were sacrificed at mid day and mid night. Blood was obtained through cardiac puncture, and leucocytes were separated. Equal amount of blood and yeast cells were incubated for 30 minutes, and smear of mixture was prepared on a clean glass slide. Slides were stained and observed in oil immersion. Percent phagocytosis was significantly ($p < 0.05$) higher (60.75 ± 1.89) during mid day as compared to mid night (50.75 ± 1.18). Phagocytic index showed non significant increase during mid night (2.44 ± 0.20) when compared to mid day (2.21 ± 0.25). Superoxide production was found to be significantly higher during night (0.357 ± 0.02) as compared to day (0.255 ± 0.02). It is suggested that diurnal variation is a part of immune system circadian oscillation.

INTRODUCTION

Immune system shows two but interrelated types of response: acquired and innate immune responses. The acquired immune response evolved in early vertebrates and allow for a stronger immune response as well as immunological memory. The innate immune response act as an initial defense mechanism against microbial growth shortly after infection occurs (Merchant *et al.*, 2003). The innate immune response of reptiles has been addressed in the literature (Koppenheffer, 1987; Freedberg *et al.*, 2008). Some reports are also available on seasonal variation in cell-mediated innate immune responses in reptiles (Munoz and Fuente, 2001). Phagocytosis is also important constituent of innate immune system and critical for the survival of organisms. The cell-mediated innate immune responses in reptiles has been addressed in literature, with reference to phagocytosis and cytotoxic response of splenic macrophages (Mondal and Rai, 1999a, b, 2001, 2002a, b), mixed leucocyte reaction and lymphocyte proliferation (Farag and El Ridi, 1985, 1986; Munoz *et al.*, 2000; Cray *et al.*, 2001; Work *et al.*, 2001; Munoz and Fuente, 2003; Burnham *et al.*, 2005; Keller *et al.*, 2005, 2006). There are a few reports on day night variation in phagocytic activity in mammals and birds (Barriga *et al.*, 2001; Berger and Slapnickova, 2003; Hriscu, 2004). With regard to ectothermic vertebrates, reports are confined to fishes only (Esteban *et al.*, 2006; Roy *et al.*, 2008). Respiratory burst function resulting in the release of reactive oxygen species (ROS) such as superoxide anion (O_2^-) from neutrophils is one of the key mechanisms of the innate immune systems. Nitroblue tetrazolium (NBT) is a yellow, water-soluble dye that can be reduced by accepting electrons in the presence of free oxygen radicals to form a blue-black water-insoluble compound known as formazan (Baehner *et al.*, 1976). Thus, the NBT reaction indirectly reflects the ROS generating activity in the cytoplasm of cells. Reptiles represent an important phylogenic group being ancestor of both birds and mammals. The objective of the present study was to explore the day night variation in phagocytosis and oxidative burst activity of leucocytes in an ophidian model, *Natrix piscator*.

MATERIALS AND METHODS

Animals

Freshwater snakes, weighing 80-120g, were obtained from a local supplier who collected these animals in the suburbs of Varanasi (28°18′N; 83°1′E). Animals were housed in vivarium (wood and wire net cages; size 50x30x30cm) containing earthen bowl filled with water. Snakes were fed on small fishes once a week. Cages were cleaned, and bowl water was changed next day following feeding. Animals were acclimatized to the laboratory conditions for two weeks, and experiments were performed. The guideline of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Ministry of Statistics and Programme Implementation, Government of India, were followed in maintenance and sacrifice of animals.

Chemicals

Culture medium (RPMI-1640), L-glutamine, gentamycin, fetal

bovine serum (FBS), and other chemicals were purchased from Himedia Laboratories Pvt. Ltd. (India). The culture medium was supplemented with 1 μL mL^{-1} gentamycin, 10 μL mL^{-1} of 200 mM L-glutamine, 10 μL mL^{-1} anti-anti (Gibco) and 5% FBS and referred to as complete culture medium.

Experiment

Animals were divided into two groups of four animals each (n = 4): one group was sacrificed at 12h mid day and other group at 12h mid night. Animals were weighed, anaesthetized and blood was collected in a heparinized syringe through cardiac puncture. Blood was processed for phagocytosis and NBT assay.

Blood phagocytosis

For phagocytic assay, the yeast cells were used as target cell. The yeast cell suspension was prepared by mixing 20mg of commercial baker's yeast (*Saccharomyces cerevisae*) in 10mL of 0.2 M PBS. The suspension was kept at 80ºC for 15min. The cells were washed three times in PBS and finally suspended in complete culture medium to get a concentration of 1x10^{8} cells mL^{-1}. Equal amount (20 μL) of blood and yeast cell suspension was mixed and incubated for 30 minutes at room temperature. Smear was prepared on a clean glass slide, air dried, fixed in methanol, stained with Giemsa, and examined under oil immersion. For each slide, a total of 100 neutrophils were examined randomly without any predetermined sequence. The phagocytic index was determined by calculating the average number of yeast cells engulfed by single neutrophil. The percent phagocytosis was calculated by dividing the number of neutrophils showing phagocytosis by 100.

NBT assay

Peripheral Bood Leucocytes (PBL) were collected from the buffy coat (the layer of PBLs between the plasma and RBCs) using a slow spin technique as described by Keller *et al*. (2005). The tubes were centrifuged at 500 rpm (42 x g) for 25 min at 8ºC. The PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 1200 rpm for10 min, the plasma was removed and the cell pellet was gently resuspended in 1mL of culture medium. NBT assay was performed following the methods of Berger and Slapnickova (2003). Leucocytes were counted and adjusted to 2x10^{6} cells mL^{-1} in complete RPMI. Cell viability was checked through trypan blue exclusion test, which exceeded 95%. 50 μL of leucocytes (10^{5} cells) was mixed with 50 μL of RPMI containing NBT (1 mg mL^{-1}) in 96 well culture plate in triplicates. One well with culture medium without cells served as blank. Plates were then incubated in CO_2 atmosphere at 25ºC for 2h, centrifuged at 700 x g, washed with PBS and fixed in 70% methanol. 20 μL of 0.1% triton X-100 was mixed in each well. The formazan crystals were dissolved by mixing 120 μl KOH (2 M) and 140 μL DMSO in each well. Optical density was measured at 620 nm with the help of ELISA plate reader (Thermo Multiscan).

Statistical analysis

Data are presented as mean ± SEM. Means were compared, and statistical difference between means was determined by Student's t-test.

RESULTS

Leucocytes obtained from snake showed day night variations in phagocytic activity. Percent phagocytosis was significantly ($p < 0.05$) higher at mid night and phagocytic index was insignificantly higher at mid night (Fig. 1). Superoxide production, as judged by NBT reduction assay, was found to be significantly ($p < 0.05$) higher during night time as compared to day (Fig. 2).

DISCUSSION

Daily rhythms in immune parameters have been documented for most species of mammals and birds studied to date. Most of these investigations, however, have focused on rhythmicity in the number of circulating immune cells and splenic lymphocytes (Haus *et al.*, 1983; Nelson *et al.*, 2002; Pelegri *et al.*, 2003; Oishi *et al.*, 2006); while few studies have examined changes in functional activity of immune cells. Diurnal rhythms in human bone marrow were first demonstrated in the work of Aardal with Laerum (1983), and Mauer (1965). Leucocytes and its subtypes, in human, vary in a circadian pattern: some show increase in daytime; while others, at night (Haus *et al.*, 1983, Suzuki *et al.*, 1997). There is now considerable evidence that magnitude of the immune

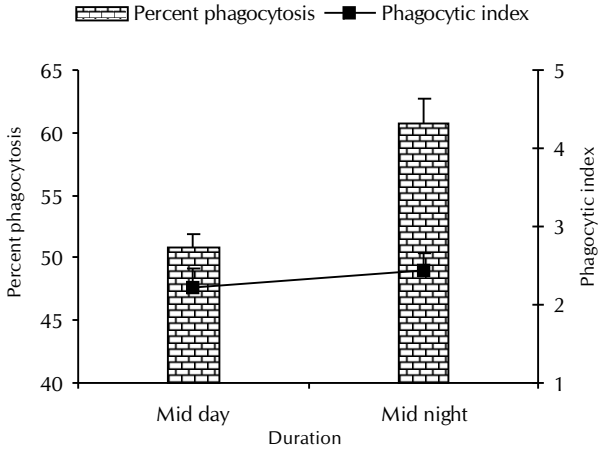

Figure 1: Day night variation in neutrophil phagocytosis in fresh water snake *Natrix piscator*

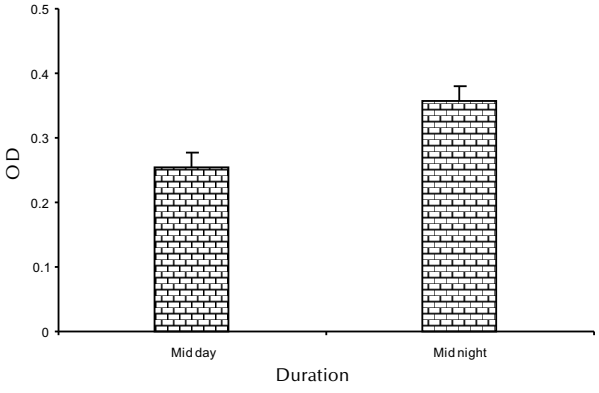

Figure 2: Day night variation in NBT reduction in fresh water snake *Natrix piscator*

response varies with time of the day. The present study in fresh water snake, *N. piscator*, demonstrates the day night rhythmicity in leucocyte phagocytosis and superoxide production. The phagocytosis was higher during dark phase than light phase. This is in agreement to that in most of the endothermic vertebrates in which phagocytic activity by polymorphonuclear granulocytes remained elevated during the dark phase, though the precise timing of acrophase varies in different animals, when the hormone melatonin secretion is high (Hriscu *et al.*, 2002–2003; Hriscu, 2004; Melchart *et al.*, 1992). Surprisingly, there are other studies in mice in which the phagocytosis is reported to be high during the light phase, for example, the maximum engulfment of carbon particles by reticuloendothelial cells in CBA mice occur during the second half of the light span (Szabo *et al.*, 1978), while phagocytes collected from different tissues of C57BL/6 mice showed peak phagocytic activity in the first half of the light span (Knyszynski and Fischer, 1981; Hayashi *et al.*, 2007). The inconsistent results pertaining to the circadian pattern of phagocytic activity are reported in humans also. The polymorphonuclear cells in one of the studies were unresponsive to the LD cycle (Bongrand *et al.*, 1988), while the same cells exhibited diurnal periodicity with peak phagocytosis at midnight in the other study (Melchart *et al.*, 1992). In ectothermic vertebrates, the knowledge is rudimentary and confined to reports in which diurnal rhythmicity of humoral innate immune functions is described in fishes, gilthead seabream, and sea bass (Esteban *et al.*, 2006). The peak complement activity in both fishes is reported during the light phase. Immune responses seem dependent on species, strain of animals, and type of immune cells and their specific functions. Neutrophil phagocytosis and oxidative burst activity in reptiles were studied by Froesce *et al*. (2005). Other work related to reptiles is confined to the study of effect of sex steroids on splenic macrophage phagocytic activity (Mondal and Rai, 1999 a, b, 2002a, b).

The innate immune activity of blood cells attains maximal value during day time. In analyzing the influence exerted by the light regimen upon innate immune functions of blood leucocyte, two distinct aspects have to be considered: the circadian structure of the rhythms and the level of the assessed functions. There are several indirect and also direct indicators that melatonin, secreted exclusively at night, would play a role in the immune function. *In vitro* studies employing pharmacological doses of melatonin (5-100μM) revealed a dose-dependent activation of phagocytic function (Rodriguez *et al.*, 1999). However, such doses are far above the physiologically available range. We may speak, more plausibly, about role of melatonin on innate immune response of *N. piscator* after further experimentation involving *in vitro* and *in vivo* melatonin administration and accessing immune parameters. In summary, these data indicate a clear cut variation in innate immune function of blood leucocytes in *N. piscator*. The findings of this study may in part explain the variations by demonstrating changes in innate immune activity of leucocytes to encounter and process antigen.

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REFERENCES

Aardal, N. P. and Laerum, O. D. 1983. Circadian variations in mouse bone marrow. *Exp. Hematol.* **11:** 792-801.

Barriga, C., Martin, M. I., Tabla, R., Ortega, E. and Rodrýguez, A. B. 2001. Circadian rhythm of melatonin, corticosterone and phagocytosis: effect of stress. *J. Pineal Res.* **30:** 180–187.

Baehner, R. L., Boxer, L. A. and Davis, J. 1976. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. *Blood.* **48:** 309–313.

Berger, J. and Slapnickova, M. 2003. Circadian structure of rat neutrophil phagocytosis. *Comp. Clin. Pathol.* **12:** 84–89.

Bongrand, P., Bouvenot, G., Bartolin, R., Tatossian, J. and Bruguerolle, B. 1988. Are there circadian variations of polymorphonuclear phagocytosis in man? *Chronobiol Int.* **5:** 81–83.

Burnham, D. K., Keall, S. N., Nelson, N. J. and Daugherty, C. H. 2005. T cell function in tuatara (Sphenodon punctatus). *Comp. Immunol. Microbiol.* **28:** 213–222.

Cray, C., Varella, R., Bossart, G. D. and Lutz, P. 2001. Altered *in vitro* immune responses in green turtles (*Chelonia mydas*) with fibropapillomatosis. *J. Zoo. Wildlife Med.* **32:** 436-440.

Esteban, M. A., Cuesta, A., Rodrýguez, A. and Meseguer, J. 2006. Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system. *J. Pineal Res.* **41:** 261–266.

Farag, M. A. and El Ridi, R. 1985. Mixed leucocyte reaction (MLR) in the snake (*Psammophis sibilans*). *Immunol.* **55:** 173-181.

Farag, M. A. and El Ridi, R. 1986. Proliferative responses of snake lymphocytes to concanavalin A. *Dev. Comp. Immunol.* **10**: 561-569.

Froese, J. M. W., Smits, J. E. G. and Wickstrom, M. L. 2005. Evaluation of two methods for measuring nonspecific immunity in tiger salamanders (*Ambystoma tigrinum*). *J. Wildlife Dis.* **41(1):** 209-217.

Freedberg, S., Greives, T. J., Ewert, M. A., Demas, G. E., Beecher, N. and Nelson, C. E. 2008. Incubation environment affects immune system development in a turtle with environmental sex determination. *J. Herpetol.* **42:** 536-541.

Haus, E., Lakatua, D., Swoyer, J. and Sackett-Lundeen, L. 1983. Chronobiology in hematology and immunology. *Am. J. Anat.* **168:** 467-517.

Hayashi, M., Shimba, S. and Tezuka, M. 2007. Characterization of the molecular clock in mouse peritoneal macrophages. *Biol. Pharm. Bull.* **30:** 621–626.

Hriscu, M., Saulea, G., Ostriceanu, S. and Baciu, I. 2002–2003. Circadian phagocytic activity in rats under light–dark and constant light regimens. *Rom. J. Physiol.* **39 & 40:** 17–26.

Hriscu, M. 2004. Circadian phagocytic activity of neutrophils and its modulation by light. *J. Appl. Biomed.* **2:** 199–211.

Keller, J. M., McClellan-Green, P. D., Lee, A. M., Arendt, M. D., Maire, P. P., Segars, A. L., Whitaker, J. D., Keil, D. E. and Peden-Adams, M. M. 2005. Mitogen-induced lymphocyte proliferation in loggerhead sea turtles: comparison of methods and effects of gender, plasma testosterone concentration, and body condition on immunity. *Vet. Immunol. Immunopathol.* **103**: 269-281.

Keller, J. M., McClellan-Green, P. D., Kucklick, J. R., Keil, D. E. and Peden-Adams, M. M. 2006. Effects of organochlorine contaminants on loggerhead sea turtle immunity: comparison of a correlative field study and *in vitro* exposure experiments. *Environ. Health Persp.* **114:** 70-76.

Knyszynski, A. and Fischer, H. 1981. Circadian fluctuations in the activity of phagocytic cells in blood, spleen, and peritoneal cavity of mice as measured by zymosan-induced chemiluminescence. *J. Immunol.* **127:** 2508-2511.

Koppenheffer, T. L. 1987. Serum complement systems of ectothermic vertebrates. *Dev. Comp. Immunol.* **11:** 279-286.

Mauer, A. M. 1965. Diurnal variations of proliferative activity in the human bone marrow. *Blood*. **26:** 1-7.

Melchart, D., Martin, P., Hallek, M., Holzmann, M., Jurcic, X. and Wagner, H. 1992. Circadian variation of the phagocytic activity of polymorphonuclear leukocytes and of various other parameters in 13 healthy male adults. *Chronobiol Int.* **9:** 35–45.

Merchant, M. E., Roche, C. M., Elsey, R. M. and Prudhomme, J. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). *Comp. Biochem. Physiol. B.* **136:** 505-513.

Mondal, S. and Rai, U. 1999. Sexual dimorphism in phagocytic activity of wall lizard's splenic macrophages and its control by sex steroids. *Gen. Comp. Endocr.* **116:** 291–298.

Mondal, S. and Rai, U. 1999. Dose-dependent effect of sex-steroids in lizard's splenic macrophage phagocytic activity. *Recent Prog. Mol. Comp. Endocrinol.* 482-488.

Mondal, S. and Rai, U. 2002. Dose and time-related *in vitro* effects of glucocorticoid on phagocytosis and nitrite release by splenic macrophages of wall lizard *Hemidactylus flaviviridis*. *Comp. Biochem. Phys. C.* **132:** 461–470.

Mondal, S. and Rai, U. 2002. *In vitro* effect of sex steroids on cytotoxic activity of splenic macrophages in wall lizard (*Hemidactylus flaviviridis*). *Gen. Comp. Endocr.* **125(2):** 264-271.

Munoz, F. Z., Galvan, A., Lerma, M. and De la Fuente, M. 2000. Seasonal changes in peripheral blood leucocyte functions of the turtle *Mauremys caspica* and their relationship with corticosterone, 17-beta- estradiol and testosterone serum levels. *Vet. Immunol. Immunop.* **77:** 27-42.

Munoz, F. Z. and Fuente, M. 2001. The immune response of thymic cells from the turtle *Mauremys caspica*. *J. Comp. Physiol. B.* **171:** 195–200.

Munoz, F. Z. and Fuente, M. 2003. Effect of migratory cycle and 17 beta-estradiol on splenic leucocyte functions in female black-headed gulls. *Pflug. Arch. Eur. J. Phy.* **445(6):** 659-664.

Nelson, R. J., Demas, G. E., Klein, S. L. and Kriegsfeld, L. J. 2002. Seasonal patterns of stress and immune fucntion and disease. Cambridge University Press, New York. pp.

Oishi, K., Ohkura, N., Kadota, K., Kasamatsu, M., Shibusawa, K., Matsuda, J., Machida, J. K., Horie, S. and Ishida, N. 2006. Clock mutation affects circadian regulation of circulating blood cells. *J. Circadian Rhythms.* **4:** 13.

Pelegri, C., Vilaplana, J., Castellote, C., Rabanal, M., Franch, A. and Castell, M. 2003. Circadian rhythms in surface molecules of rat blood lymphocytes. *Am. J. Physiol-Cell. Ph.* **284:** C67-C76.

Rodriguez, A. B., Marchena, J. M., Nogales, G., Duran, J. and Barriga, C. 1999. Correlation between the circadian rhythm of melatonin, phagocytosis, and superoxide anion levels in ring dove heterophils. *J. Pineal Res.* **26:** 35–42.

Roy, B. and Rai, U. 2008. Diurnal variation in phagocytic activity of splenic phagocytes in fresh water teleost *Channa punctatus*: melatonin and its signaling mechanism. *J. Endocrinol.* **199:** 471-480.

Suzuki, S., Toyabe, S. and Moroda, T. 1997. Circadian rhythm of leucocytes and lymphocyte subsets and its possible correlation with the function of the autonomic nervous system. *Clin. Exp. Immunol.* **110:** 500-508.

Szabo, I., Kovats, T. G. and Halberg, F. 1978. Circadian rhythm in murine reticuloendothelial function. *Chronobiologia.* **5:** 137-143.

Work, T. M., Rameyer, R. A., Balazs, G. H., Cray, C. and Chang, C. P. 2001. Immune status of free-ranging green turtles with fibropapillomatosis from Hawaii. *J. Wildlife Dis.* **37:** 574-581.