Journal of Stress Physiology & Biochemistry, Vol. 8 No. 2 2012, pp. 145-151 ISSN 1997-0838

ORIGINAL ARTICLE

Photoperiodic stress on nitrite production by splenic macrophages in fresh-water snake *Natrix piscator*

Tripathi Manish Kumar and Singh Ramesh

Department of Zoology, Udai Pratap Autonomous College, Varanasi -221002, Uttar Pradesh, India

Phone: +91 9415687648 Fax: +91 542 2282399

**E-mail:upcrsingh@yahoo.com*

Received March 21 2012

Changes in day length enhance or suppress component of immune function in individuals of several species. The purpose of the present experiment was to study the role of photoperiodic manipulation on the nitric oxide production by splenic macrophages in the fresh-water snake, *Natrix piscator*. To study effect of photoperiod, animals were subjected to 24 hour continuous light and continuous dark for 30 days. Animals kept in natural day length served as control. At termination of experiments, animals were sacrificed, and spleen was excised. Macrophages were incubated for 24 hours and nitric oxide production was measured by measuring the nitrite concentration. Nitrite production was significantly decreased to the cultures obtained from the animals kept under continuous light. No change in nitrite concentration was found in animals kept under continuous dark, when compared to the animals kept under natural day length. The possible role of decreased melatonin synthesis in light is suggested to decrease the nitric oxide production.

Key words: nitric oxide/ photoperiod/ snake/ splenic macrophage

ORIGINAL ARTICLE

Photoperiodic stress on nitrite production by splenic macrophages in fresh-water snake *Natrix piscator*

Tripathi Manish Kumar and Singh Ramesh

Department of Zoology, Udai Pratap Autonomous College, Varanasi -221002, Uttar Pradesh, India
Phone: +91 9415687648 Fax: +91 542 2282399
**E-mail:upcrsingh@yahoo.com*

Received March 21 2012

Changes in day length enhance or suppress component of immune function in individuals of several species. The purpose of the present experiment was to study the role of photoperiodic manipulation on the nitric oxide production by splenic macrophages in the fresh-water snake, *Natrix piscator*. To study effect of photoperiod, animals were subjected to 24 hour continuous light and continuous dark for 30 days. Animals kept in natural day length served as control. At termination of experiments, animals were sacrificed, and spleen was excised. Macrophages were incubated for 24 hours and nitric oxide production was measured by measuring the nitrite concentration. Nitrite production was significantly decreased to the cultures obtained from the animals kept under continuous light. No change in nitrite concentration was found in animals kept under continuous dark, when compared to the animals kept under natural day length. The possible role of decreased melatonin synthesis in light is suggested to decrease the nitric oxide production.

Key words: nitric oxide/ photoperiod/ snake/ splenic macrophage

Evidence that photoperiod can regulate important immune cell functions in mammals led us to study the photoperiodic influence on nitric oxide production by splenic macrophages in fresh-water snake *Natrix piscator*. Spleen is a secondary lymphoid organ having meshwork of reticular cells and rich supply of macrophages, and it is supposed to serve mainly as a filter for antigenic particles, parasitized blood cells and immune complexes in blood and subsequent phagocytosis. Splenectomy suppresses the humoral response totally or partially, demonstrating the functional importance of the spleen in reptiles (Kanakambika and Muthnukkaruppan, 1972; Hussein et al., 1979). Nitric Oxide (NO), produced endogenously from L-Arginine by nitric oxide synthetases, plays an important role in many physiological processes including vascular regulation, immune responses, and neural communication. NO is extremely unstable and undergoes repaid oxidative degradation to nitrite (NO_2^-) and nitrate (NO_3^-), which can be spectrophotometrically determined. 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). Some reports are also available on seasonal variation in cell-mediated innate immune responses in reptiles (Garcia and Fuente, 1991; Zapata et al., 1992; Munoz et al., 2000; Munoz and Fuente, 2001). Study regarding photoperiodic manipulation and nitric oxide production is lacking in fresh-water snake, hence, present study was undertaken to study the effect of photoperiodic manipulation on nitric oxide production by splenic macrophages in this species.

MATERIALS AND METHODS

Animals

Male fresh-water snakes, weighing 80-120g, were obtained from a local supplier who collected these animals in the suburbs of Varanasi (28^0 18'N; 83^0 1'E). Animals were brought to the unconditioned laboratory. Animals were housed in vivarium (wood and wire net cages; size 50x30x30 cm). Each cage had an earthen bowl (4L capacity) filled with water and accommodate 4-5 snakes. Snakes were fed on small fishes once a week. Cages were cleaned, and bowl water was changed next day following feeding. The guideline of the committee for the purpose of control and supervision of experiment on animals (CPCSEA), Ministry of Statistics & Programme Implementation, Government of India, were followed in maintenance and sacrifice of animals.

Experiment

Animals were divided into three groups: Group one animals were maintained in natural light dark cycle (10L:14D) and served as control, group two animals, in continuous light (24L), and group three animals, in continuous dark (24D) for 30 days. Animals were sacrificed and spleen was isolated aseptically. Spleen was macerated through a nylon strainer of pore size <100 µm into complete culture medium (2 ml per spleen) to get single cell suspension under a sterile laminar flow hood. Spleno-somatic index (SSI: spleen weight per 100 g body weight) was calculated. Spleen cellularity (number of cells mg^{-1} tissue) was determined with help of hemocytometer and light microscope.

Nitrite Assay

100 µl of spleen cell suspension or standard KNO_3 was added to 96 well culture plate in triplicate and incubated for two hours at 25 ^{0}C. After incubation, adhered macrophages were washed twice with phosphate buffered saline and 100 µl freshly prepared culture medium was added in each well. Plate was again incubated for 24 hours in CO_2 atmosphere at 25 ^{0}C. Plate was centrifuged at 200g for 10 minutes. 50µl of supernatant and 50 µl of Griess Reagent (25 µl of 1% sulphanilamide prepared in 3 N HCl and 25 µl of 0.1% N-naphthyl ethylenediamine prepared in distilled water) was mixed in microplate and after 10 minutes absorbance was measured at 540 nm with the help of ELISA plate reader (Thermo).

Statistical analysis

Nitrite concentration (µM) was calculated for each sample. Data are presented as mean ± SEM. Means were compared, and statistical difference between means was determined by Student's t-test.

RESULTS

Spleno-somatic index was increased in the animals kept under continuous dark, though insignificantly. Spleen cellularity was significantly increased in the animals kept under 24 hour dark. Surprisingly cellularity was also increased in the animals kept in continuous light (Fig. 1 and 2). Nitric oxide production, as measured by nitrite concentration, was significantly decreased ($p<0.05$) to the cultures obtained from the animals kept under

continuous light. There was no significant difference in nitric oxide production by macrophages obtained from animals kept under continuous dark, when compared to animals kept under natural day length (Fig. 3).

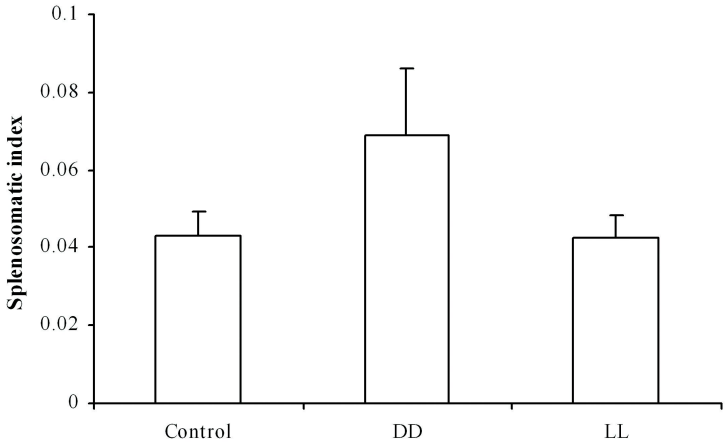

Figure 1. Effect of photoperiod on Spleno-somatic index in the fresh-water snake, *Natrix piscator*. (LD – Natural daylength; DD – Complete dark; LL – Complete light).

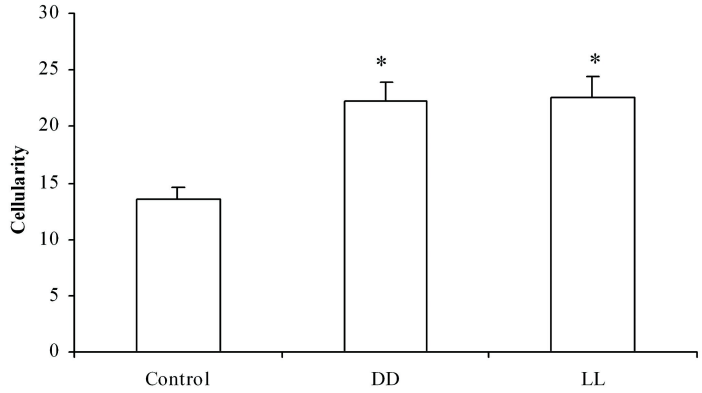

Figure 2. Effect of photoperiod on spleen cellularity in the fresh water-snake, *Natrix piscator*. (LD – Natural daylength; DD – Complete dark; LL – Complete light) (***** $p < 0.05$).

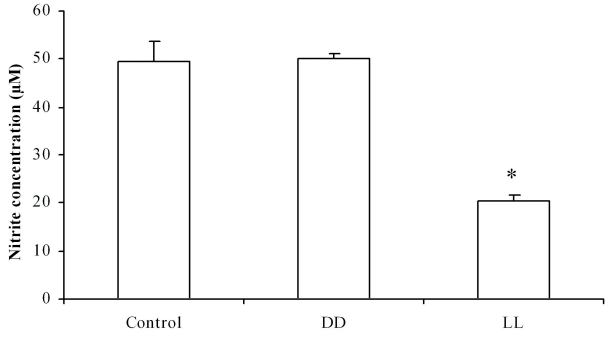

Figure 3 Effect of photoperiod on nitric oxide production by splenic macrophages in the fresh-water snake, *Natrix piscator*. (LD – Natural daylength; DD – Complete dark; LL – Complete light) (* $p < 0.05$).

DISCUSSION

Macrophages play a critical role in the induction and expression of many innate and acquired immune responses and provide a front-line host defense against invading microbes and newly formed tumor cells (Adams and Hamilton, 1984). Their primary functions are destruction of pathogens either directly by phagocytosis or indirectly by secretion of a variety of non-specific cytotoxic substances such as reactive nitrogen/oxygen intermediates (RNI/ROI) and cytokines, TNF-α and IL-1. Macrophages also play an important role in controlling the proliferation of intracellular micro-organisms. This function is important during the onset of the immune response, when macrophages, alone or in collaboration with natural killer (NK) cells, are able to inhibit the proliferation of and can kill pathogenic intracellular micro-organisms at an early stage of the infection (Bancroft et al., 1991). Reactive nitrogen intermediates (RNI), produced by macrophages and other cell types in response to IFN-y, or IFN-y plus tumour necrosis factor-a (TNF-a) and interleukin-1 (IL-1), have been shown to play an important role in killing of pathogens (Flesch and Kaufmann, 1991). In the present study splenosomatic index was insignificantly higher in animals kept under continuous dark but spleen cellularity was increased in both groups kept either under dark or light. The change in spleen mass and cellularity is possibly due to changes in melatonin secretion by pineal gland because melatonin has been suggested to have been immunomudulatory roles (Pelham et al., 1972; Pang and Ralph, 1975). Nitric oxide production was significantly reduced in the animals kept under continuous light which might be result of reduced synthesis of melatonin in light. In summary, the result of the present study shows that continuous light exposure to fresh-water snake reduces nitric oxide production by splenic macrophages.

ACKNOWLEDGEMENT

Financial grant (SR/SO/AS-15/2005) from DST, New Delhi to RS is gratefully acknowledged. MKT is indebted to CSIR, New Delhi for award of JRF.

REFERENCES

Admas, D. O. and Hamilton, T. A. (1984) The cell biology of macrophage activation. *Ann Rev Immunol*., **2**, 283-289.

Bancroft, G. J., Schreiber, R. D. and Unanue, E. (1991) Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the Scid mouse. *Immunol Rev*., **124**, 5-24.

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

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

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.

Flesch, I. E. A. and Kaufmann, S. H. E. (1991) Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. *Infect Immunol*., **59**, 3213-3218.

Garcia, S. and De la Fuente, M. (1991) Seasonal variations in the activity of in vitro peripheral blood granulocytes in the turtle *Mauremys caspica*. *Comp Biochem Physiol*., **100**, 697–702.

Hussein, M. F., Badir, N., El-Ridi, R. and El Deeb, S. (1979) Effect of seasonal variations on lymphoid tissues of the lizard *Scincus scincus*. *J Exp Zool*., **209**, 91.

Kanakambika, P. and Muthnukkaruppan, V. (1972) Effect of splenectomy on the immune response in the lizard, *Calotes versicolor*. *Experientia*., **28**, 1225-1226.

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 Immunop*., **103**, 269-281.

Keller, J. M., McClellan-Green, P. D., Kucklick, J. R., Keil, D. E., 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.

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. (2001) In vitro effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, *Hemidactylus flaviviridis*. *Comp Biochem* Physiol., **129**, 391–398.

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* Endocrinol., **125**, 264-271.

Munoz, F. Z., Galvan, A., Lerma, M., 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**, 659-664.

Pang, S. F. and Ralph, C. L. (1975) Pineal and serum melatonin activities at mid-day and mid-night following pinealectomy or castration in male rats. *J Exp Zool*., **194**, 275–280.

Pelham, R. W., Ralph C. L. and Campbell, I. M. (1972) Masspectral identification of melatonin in blood. *Biochem Biophys Res Commun*., **46**, 1236–1241.

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.

Zapata, A. G., Varas, A. and Torroba, M. (1992) Seasonal variations in the immune system of lower vertebrates. *Immunol Today*., **13**, 142-147.