The Bioscan 7(2) : 197-200, 2012
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

EFFECT OF PHOTOPERIOD AND MELATONIN ON SPLENOCYTE PROLIFERATION IN FRESHWATER SNAKE, *NATRIX PISCATOR*

MANISH KUMAR TRIPATHI* AND RAMESH SINGH
Department of Zoology, Udai Pratap Autonomous College, Varanasi - 221 002, U.P., INDIA
E-mail: upcrsingh@yahoo.com

KEY WORDS
Photoperiod
Melatonin
Splenocyte proliferation
Snake

Received on :
22.03.12

Accepted on :
19.05.12

***Corresponding author**

ABSTRACT
The purpose of the present experiment was to investigate the effect of different photoperiod regimens on splenocytes proliferation in fresh-water snake *Natrix piscator*. To study the effect of photoperiod on lymphocyte activities, different photoperiod regimens were used. These were: constant light (24D) and dark (24L) for 30 days. Animals kept under natural day length served as control. Splenocyte proliferation was studied colorimetrically using MTT 3-(4, 5-dimethylthiozol-2-yl)-2, 5 diphenyl tetrazolium bromide]. Mitogens used were T-cell mitogen, concanavalin A (ConA; 5 and 10μg mL^{-1}) and phytohemagglutinin (PHA; 5 and 10μg mL^{-1}). The direct effect of melatonin on immune cells was evaluated by adding melatonin to the cultures obtained from animals kept in different photoperiod conditions. The results of these studies suggest that splenic lymphocytes from 24D animals have increased mitogen-induced proliferative response, while those from 24L animals have decreased Proliferative response. Further, *in vitro* melatonin caused significant enhancement of proliferation of splenocytes obtained from 24L treated animals. Our results emphasize the effect of photoperiod and melatonin on proliferative activity of splenocytes in this species.

INTRODUCTION

The neuroendocrine and immune systems help an organism cope with changing environmental conditions. In recent years, melatonin, a neural hormone synthesized and secreted primarily by the pineal gland (Pelham *et al.*, 1972; Pang and Ralph, 1975), has been suggested to have immunomodulatory roles. The involvement of melatonin in the establishment of a pineal-immune system regulatory axis is currently of interest (Guerrero and Reiter, 1992; Liebmann *et al.*, 1997). In mice, melatonin treatment *in vivo* increased concanavalin A (Con A)-induced lymphocyte proliferation but decreased lipopolysaccharide (LPS) - induced lymphoproliferation (Champney *et al.*, 1997).

Recent approaches in studying the mechanism of pineal gland regulation of immune function have focused on photoperiod or day length. Light-dark stimuli provide adequate environmental information necessary for physiological and behavioral adaptive changes. Pinealectomy seems to prevent photoperiodic responses in many mammalian species (Goldman, 1983). Melatonin production reaches its maximum level at the midpoint of the dark phase and its minimum level at the midpoint of the light phase (Lynch, 1971). Therefore, the pineal gland appears to translate environmental cues into melatonin secretions. In a study, significant elevation in splenocyte proliferation to ConA was noticed in mice grown under short-day conditions compared with mice grown under long-day conditions (Demas *et al.*, 1996). Mitogen induced lymphocyte proliferation is widely used functional *in vitro* assessment of innate immune response. Study, regarding photoperiodic manipulation and melatonin effect on splenocytes, is lacking in reptiles, though reptiles represent important phylogenic group being ancestor of both birds and mammals. Hence considering the importance of reptiles, present study was undertaken to elucidate the role of photoperiod and melatonin on splenocytes proliferation in an ophidian, *Natrix piscator*.

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 (28º18'NL; 83º1'EL). Animals were housed in vivarium (wood and wire net cages; size 50x30x30cm). 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 and Programme Implementation, Government of India, were followed in maintenance and sacrifice of animals.

Chemicals

MTT, ConA and PHA were purchased from Sigma Chemicals. Culture medium (RPMI-1640), lymphocyte separation medium (HiSep), L-glutamine, gentamycin, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), 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 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. The animals from each group 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.

Splenic Lymphocyte proliferation assay

Splenic lymphocytes were isolated by density gradient centrifugation using HiSep (Density 1.077 g mL^{-1}). Spleen cell suspension was overlaid on equal volume of HiSep in tubes and centrifuged at 400 x g for 30 min with brakes off at 8ºC. Following centrifugation, lymphocyte fraction at the interface between medium and HiSep was carefully aspirated, washed three times with PBS, counted and assessed for viability on a hemocytometer through trypan blue exclusion test. Viable cells ($>$ 95%) were adjusted to $2x10^6$ cells mL^{-1} in complete culture medium.

Basal as well as mitogen (ConA and PHA)-induced lymphocyte proliferation was assessed *in vitro* following the methods of Berridge *et al*. (2005). Stock solution of mitogen was made in 0.2 M PBS (pH 7.2) at a concentration of 1 mg mL^{-1}. Further dilution was made in culture medium. Flat bottom 96 well culture plates were used. Mitogens (50 μl; final concentration 5 and 10 μg mL^{-1}) was added, and then 50 μl cell suspension ($2x10^6$ cells mL^{-1}) was seeded into well of culture plate. To study spontaneous proliferation, 50 μl cell suspension was seeded into well of culture plate along with 50 μl of mitogen-free culture medium. Additional well in triplicate contained only 100 μl of culture medium and served as blank. Plates were incubated in humidified CO_2 atmosphere at 25ºC for 48h. Following incubation, 10 μl of MTT reagent (5 mg mL^{-1}) was added to each well and plates were again incubated overnight in humidified CO_2 atmosphere at 25ºC. After incubation, the supernatant was aspirated, and 100 μl of DMSO was added to each well to solubilize the formazan crystals. Absorbance was measured at 570 nm with the help of ELISA plate reader (Multiscan). All samples were assayed in triplicates.

Statistical analysis

Means were compared by ANOVA followed by Newman Keuls multiple range test. All mean differences among treatment groups were considered significant if $p<0.05$.

RESULTS

Significant difference due to different photoperiod regimens was found in lymphocyte proliferations from spleen. This result occurred in response to either Con A or PHA. Splenocytes, obtained from animals kept in 24D for 30 days, showed enhanced proliferation in response to mitogen Con A as compared to proliferation of cells either from control (10L:14D) or 24L animals (Fig. 1). When another mitogen PHA was used, result again confirmed the enhanced proliferation of splenocytes from animals kept in 24D (Fig. 2). Melatonin *in vitro* significantly increased splenocyte proliferation from 24L treated animals but not in control or 24D treated animals (Fig. 3).

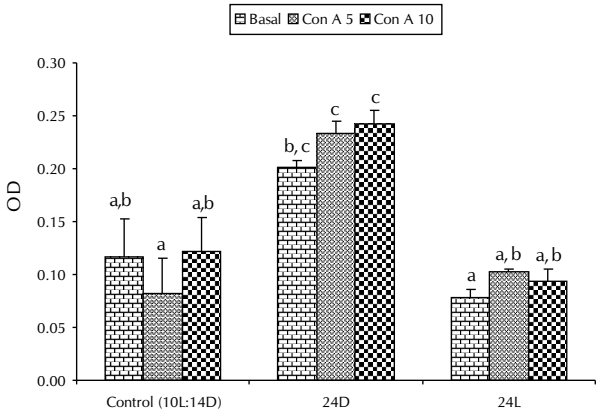

Figure 1: Effect of photoperiod on ConA induced splenocyte proliferation in fresh-water snake *Natrix piscator*

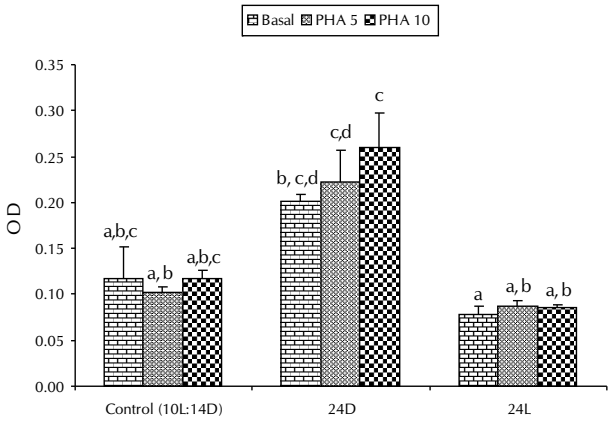

Figure 2: Effect of photoperiod on PHA induced splenocyte proliferation in fresh-water snake *Natrix piscator*

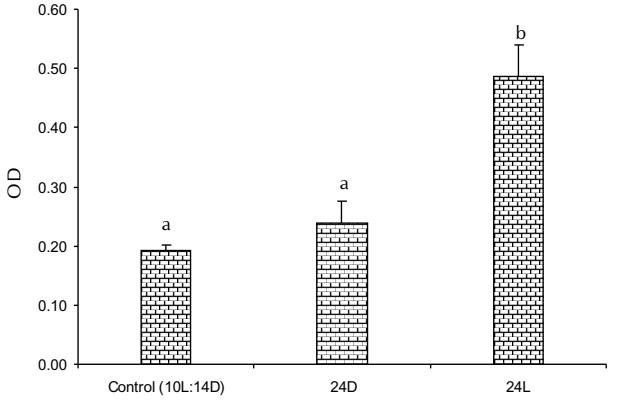

Figure 3: Effect of *in vitro* melatonin on splenocyte proliferative response to different photoperiods in fresh-water snake *Natrix piscator*

DISCUSSION

In the present study continuous dark was found to enhance mitogen-induced splenocyte proliferation in fresh-water snake *Natrix piscator*. This is in agreement with Kliger *et al*. (2000), who reported that increasing periods of darkness stimulate

mitogen-induced splenocyte proliferation in male broiler chickens. Dobrowsolska and Gromadzka-Ostrowska (1984), also reported that splenocytes extracted from male deer mice that received 8 h L : 16 h D/day for an 8-wk period exhibited greater proliferation to Con A than splenocytes from mice that received 16 h L : 8 h D/day. Furthermore, Demas and Nelson (1996) showed that deer mice grown in short days exhibited larger spleen size and higher antibody titers than deer mice maintained in long days. However, higher proliferative response has been reported in long day Siberian hamsters than short day hamasters (Demas *et al*. 2003). The possible explanations for the effects of photoperiod on mitogen-induced lymphocyte proliferation can be deduced from Champney *et al*. (1997) who suggested that melatonin can disproportionately alter the number of blood and splenic lymphocytes, or it can modify the intrinsic mitogenic activity of each lymphocyte. The result from the present study indicates that a modification of mitogenic activities and/or change in cell population has occurred which resulted in enhanced splenocyte proliferation.

In addition to photoperiod having a direct effect on mitogen-induced splenocyte proliferation, it also enhanced the sensitivity of lymphocytes to melatonin stimulation *in vitro*. Splenic lymphocytes obtained from snakes kept in 24h light were more responsive to melatonin stimulation than lymphocytes from snakes kept in 24h dark. Colombo *et al*. (1992) have suggested that spleen cells obtained at night have a high sensitivity to melatonin, whereas cells obtained in the morning have a low sensitivity because of up- and down-regulation of receptors, respectively. Spleen cells to which no melatonin was added had a greater proliferative response to Con A when removed from animals and stimulated in the middle of the dark cycle compared with untreated cells taken from animals and stimulated during the middle of the light cycle (Drazen *et al*., 2001). Pelham *et al*. (1972) reported that a low level of melatonin in chickens grown under constant lighting could allow for an up-regulation of melatonin receptor expression. Therefore lymphocytes from animals in constant lighting are more sensitive when incubated with melatonin. Kriegsfeld *et al*. (2001) have reported that splenocyte proliferation in response to the T-cell mitogen Con A was enhanced by the addition of melatonin *in vitro* compared to cultures that did not receive melatonin in male voles. However, photoperiod and reproductive condition did not significantly influence splenocyte proliferation under basal conditions, or in response to mitogen treatment. These findings suggest that melatonin enhances immune function in male voles, and this responsiveness to exogenous melatonin is not modulated by photoperiod or reproductive condition. This might be because of endogenous changes in the duration of melatonin secretion may not lead to pronounced changes in immune function in prairie voles, whereas exogenous (*i.e.* higher concentrations) melatonin may be necessary to enhance immune function in this species.

In summary, our results indicate that the 24h dark photoperiod regimen, indirectly through melatonin, enhances splenic immune functions of fresh-water snake when compared with 24 h light or 10L: 14D photoperiod regimen. These results suggest the important role that photoperiod plays in affecting the immune response. Furthermore, splenic lymphocytes from fresh-water snake receiving constant lighting are more sensitive to melatonin *in vitro* than splenic lymphocytes from fresh-water snake receiving 24h dark or 10L: 14D photoperiod.

ACKNOWLEDGEMENT

Financial Grant (SR/ SO/ AS – 15/ 2005) from Department of Science and Technology, New Delhi, Govt. of India to RS is gratefully acknowledged. MKT is indebted to Council of Scientific and Industrial Research, India for award of Junior Research Fellowship.

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