Chapter-5

EFFECT OF IN VITRO TESTOSTERONE ON IMMUNE FUNCTIONS OF BLOOD LEUCOCYTES IN THE FRESH-WATER SNAKE, NATRIX PISCATOR
5.1. INTRODUCTION

Sex differences in immunocompetence are well established in vertebrates: males typically exhibit reduced cell-mediated (e.g., proliferation of immune cells, inflammatory responses, cytokine production) and humoral (e.g., antibody production) immune responses, as compared to females (Schuurs and Verheul, 1990). Males and females also differ in susceptibility to various diseases. For example, males are more susceptible than females to a variety of parasitic infections (review Zuk and Mc Kean, 1996), certain types of cancer, e.g., lymphomas and leukemias (Billingham, 1986), and viral infections of the central nervous system (Barna et al., 1996). Humoral immune responses (i.e., antibody production by B-cells) are typically elevated in females, as compared with males; females of various species display higher IgM, IgG, and IgA concentration than males, and are also better able to mount both primary and secondary antibody responses to antigenic challenge than males (Butterworth et al., 1967; Eidinger and Garrett, 1972; Schuurs and Verheul, 1990). Conversely, females of many species more readily produce immune responses against ‘self’ tissues and are, therefore, more likely to develop autoimmune diseases than males (Olsen and Kovacs, 1996).

Sex differences in immune function are mediated, in part, by the suppressive effects of testosterone on the immune system (Alexander and Stimson, 1988; Zuk et al., 1995; Olsen and Kovacs, 1996; Hillgarth et al., 1997), as gonadectomized male rodents display immune responses that are similar to those of conspecific females (i.e. higher than gonadally intact males). Gonadectomized male rodents have elevated antibody production, increased immunological tolerance to skin graft, and heavier lymphoid organs (e.g., thymus, spleen, and lymph nodes) than gonadally intact males (Grossman, 1984; Schuurs and Verheul, 1990). These data illustrate that immunosuppression by testosterone is one proximate mechanism underlying increased susceptibility of male mammals to infection.
Administration of testosterone has been shown to reduce both humoral and cell-mediated immune responses (Grossman, 1984). Laboratory studies on rodents have demonstrated that males are more susceptible to infection than females, and this difference is related to sex steroid hormones (Tiuria et al., 1995; Klein et al., 1999, 2000). Lymphocyte proliferation response, a promising marker of host immunity, has been reported to exhibit variability during various physiological and reproductive phases in different animal species (Lacetera et al., 2005; Bilbo and Nelson, 2000), and that has been attributed to sex hormones.

Although sex differences in immune function and susceptibility to infection have also been reported in non-mammalian species, the precise role of sex steroids has not been adequately addressed (Klein, 2000). Few studies have suggested that testosterone may induce atrophy of lymphoid organs and reduced immune responsiveness in birds, reptiles, and fish (Zuk et al., 1995; Hillgarth et al., 1997; Hasselquist et al., 1999; Evans et al., 2000). In reptiles, a diversified and phylogenetically important group, being intermediate between heterotherms and homeotherms, the only study by Mondal and Rai (1999, 2001) has reported sex steroids-modulation in splenic macrophage phagocytosis and cytotoxic responses in wall lizard. There is no report on this aspect in an ophidian species, which have lacertilian lineage. Hence, this study was performed in the fresh-water snake, which is the first report of its kind. In this study, effects of in vitro testosterone on phagocytosis and cytotoxic responses of peripheral blood leucocytes as well as on mitogen induced lymphocyte proliferation were studied.

5.2. MATERIALS AND METHODS

5.2.1. Animals

Fresh-water 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). As sexual dimorphism is evident, only males were used in this study during April and May, when animals were reproductively inactive (Haldar and
Pandey, 1989). Animals were brought to the unconditioned laboratory experiencing natural ambient environmental conditions (Max. Temp. 36 – 39, Min. Temp. 24 – 25 °C; photoperid 12.5 – 13.20 hr; Relative humidity 40 – 45 %). Animals were housed in cages (size 50x30x30 cm). Each cage had wooden floor and frame with wire net sides, one side being window. Each cage had an earthen bowl (4 L capacity) filled with water to 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. Animals were acclimated to the laboratory conditions for two weeks, and experiments were performed. 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, were followed in maintenance and sacrifice of animals.

5.2.2. Chemicals

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide], NBT (Nitroblue Tetrazolium salt), mitogens (PHA, Phytohemagglutinin, ConA, Concanavalin A, Lipopolysaccharide, LPS) and testosterone 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.

5.2.3. Isolation of leucocytes

Animals were sacrificed under mild anaesthesia. Blood was sampled through cardiac puncture in heparinized tubes. Peripheral Blood Leucocytes (PBLs) 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 42xg for 25 min at 8°C. The PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 200xg for 10 min, the plasma
was removed and the cell pellet was gently resuspended in 1 ml of complete culture medium. Cell viability was checked light microscopically through trypan blue exclusion test, that exceeded 95%, cell count was adjusted at desired level.

5.2.4. NBT assay

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 culture medium. 50 µl of leucocyte cell suspension (10^5 cells) was seeded with 50 µl of different concentrations of testosterone (final concentration of 10, 100 and 1000 ng ml^{-1}) in wells of culture plate (96 well) and incubated for four hours in humidified CO_2 atmosphere at 25° C, following which 50 µl of culture medium containing NBT (1 mg ml^{-1}) was mixed. Well with Culture medium (150 µl) without cells served as blank. All assays were performed in triplicates per snake. Plate was then incubated for 2 hr, centrifuged at 700xg, washed with PBS and fixed in 70% methanol. 20 µl of 0.1% triton X-100 was mixed in each well. The formazan crystals, present inside the cells, were dissolved by mixing 120 µl KOH (2 M) and 140 µl DMSO. Optical density was measured at 620 nm with the help of ELISA plate reader (Thermo Multiscan). Following blank substraction, triplicates were averaged.

5.2.5. Nitrite assay

Nitrite content was measured by the method of Ding et al. (1988). Briefly, 50 µl of leucocytes (1x10^5 cells) and 50 µl of different testosterone concentrations (final concentration of 10, 100 and 1000 ng ml^{-1}) were added to each well, and plates were incubated in humidified CO_2 atmosphere at 25° C for 24 hours. Following incubation, plates were then centrifuged at 200xg, and supernatant was collected. Equal volume (50 µl) of supernatant (macrophage conditioned medium) and Griess reagent (1% sulfanilamide in 3N HCl and 0.1% naphthylendiamine dihydrochloride in distilled water) are mixed, and the optical density of solution was measured at 540 nm with the help of ELISA plate reader (Thermo Multiscan). Culture medium without cells served as
blank. All assays were performed in triplicates. Following blank substraction, the triplicates were averaged.

5.2.6. Lymphocyte proliferation assay

Blood lymphocytes were isolated by density gradient centrifugation using HiSep (Density 1.077 g ml⁻¹). Whole blood was overlaid on equal volume of HiSep and centrifuged at 400xg for 30 min with brakes off at 8°C. 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 2x10⁶ cells ml⁻¹ in complete culture medium.

Basal as well as mitogen-induced in vitro lymphocyte proliferation was assessed using colorimetric assay based on tetrazolium salt (MTT) following the method 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. To study basal or spontaneous proliferation, 50 µl lymphocyte was seeded into well of culture plate along with 150 µl of mitogen-free culture medium. Additional well contained only 200 µl of culture medium and served as blank. To study mitogen induced proliferation, 50 µl of different mitogens (Con A, PHA and LPS, final concentration of each at 10 µg ml⁻¹; this concentration was found effective in inducing proliferation. as in Chapter -1), 50 µl leucocytes (2x10⁶ cells ml⁻¹) and 100 µl of culture medium — total volume 200 µl—were seeded into well of culture plate. To study effect of in vitro testosterone, 50 µl of different mitogens, 50 µl cell suspension (2x10⁶ cells ml⁻¹) and 100 µl of culture medium containing different concentrations of testosterone (having final concentration of 10, 100 and 1000 ng ml⁻¹) were added to each well. A set of wells that did not contain testosterone served as control. All samples were assayed in triplicates. Plates were incubated in humidified CO₂ atmosphere at 25°C for 48 hr. Following incubation, 20 µl of MTT reagent (5 mg ml⁻¹) was added to each well, and plates were again incubated overnight in humidified
CO₂ atmosphere at 25⁰C. After incubation, the plates were centrifuged at 400xg for 10 min at 8⁰C. 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 (Thermo Multiscan). Following blank subtraction, the triplicates are averaged.

5.2.7. Statistical Analysis

Data are presented as mean ± SEM. Means were compared by Analysis of Variance (ANOVA) followed by Newman Keuls multiple-range test.

5.3. RESULTS

5.3.1. NBT reduction

There was no change in super oxide production — as judged by NBT reduction — at in vitro testosterone concentrations below 1 ng ml⁻¹ (data are not shown). Hence, higher concentrations of testosterone, 1, 10, 100 and 1000 ng ml⁻¹ were used. 1 ng ml⁻¹ testosterone concentration had no effect, while NBT reduction was decreased significantly (p<0.05) at 10, 100 and 1000 ng ml⁻¹ concentrations. The decrease was maximum at 1000 ng ml⁻¹ testosterone concentration (Fig. 5.1).

5.3.2. Nitrite release

As in NBT reduction, there was also no change in nitrite release at in vitro testosterone concentrations below 10 ng ml⁻¹ (data were not shown). Hence, higher concentrations of testosterone, 10, 100 and 1000 ng ml⁻¹ were used in this assay also. Nitrite release was significantly (p<0.05) reduced by in vitro testosterone at 100 ng ml⁻¹, but not either at 10 or at 1000 ng ml⁻¹ concentration.

When the leucocytes were pre-incubated with testosterone receptor antagonist, cyproterone acetate (CPA), the decrease in Nitrite release was alleviated, as Nitrite release was comparable to that of control leucocytes incubated in medium alone (Fig. 5.2).
Fig. 5.1 Effect of *in vitro* testosterone on NBT reduction by leucocytes in the fresh-water snake, *Natrix piscator*. The error bars bearing the same superscript do not differ significantly. Experiment was repeated twice with different animals to check reproducibility, and a result of one is presented (N= 5-6).

Fig. 5.2 Effect of *in vitro* testosterone (T 0 to 1000 – Testosterone 0 to 1000 ng ml\(^{-1}\)) on nitrite release by leucocytes in the fresh-water snake, *Natrix piscator*. Effect of testosterone receptor antagonist cyproterone acetate (CPA, 100 ng ml\(^{-1}\)) is also shown. The error bars bearing the same superscript do not differ significantly.
**Fig. 5.3** Effect of *in vitro* testosterone on mitogens (Con A, PHA and LPS – Concanavalin A, Phytohemagglutinin and Lipopolysaccharide, respectively) induced lymphocyte proliferation in the fresh-water snake, *Natrix piscator*. (final concentration of mitogens; Con A – 10 μg ml⁻¹, PHA - 10 μg ml⁻¹, LPS - 20 μg ml⁻¹). The error bars bearing the same superscript do not differ significantly.

### 5.3.3. Lymphocyte proliferation

No significant difference was obtained in basal as well as mitogen-induced lymphocyte proliferation when the lymphocytes were treated with different concentrations of *in vitro* testosterone (Fig. 5.3).

### 5.4. DISCUSSION

The immunoregulatory roles of testosterone have been assessed mainly through four different means: (1) comparing male and female differences in immunocompetence; (2) examining associations between circulating testosterone levels and measurements of immune function, such as size of immune organs or leukocyte counts in healthy or parasitized animals; (3) experimentally manipulating testosterone levels through castration or
supplementation and looking at their effects on immunocompetence; and (4) performing in vitro analyses of immune–endocrine interactions.

In present investigation we have studied in vitro effects of testosterone on immune parameters of peripheral blood leucocytes. Super oxide production, as measured by NBT reduction assay, was significantly suppressed at 1000 ng ml⁻¹ testosterone concentration: similarly nitric oxide production, as measured by nitrite assay, was also reduced in testosterone concentration dependent manner. This is in accordance with the observation of Mondal and Rai (1999, 2002) who reported in vivo and in vitro suppressive effect of testosterone on lizard splenic macrophage activity.

The sexual dimorphism has been shown in snakes by Saad et al. (1986), where normal female blood lymphocytes respond relatively more vigorously than male lymphocytes and the sex steroids were assumed to be responsible for the sex associated immune differences. The relationship between sex steroids and specific immune responses is reported in a number of reptilian species (Laceta and Zapata, 1986; Saad et al., 1986; Zapata et al., 1992; Mondal and Rai, 1999).

The present investigation suggests that testosterone modulates nitrite production via a receptor-mediated system, as treatment with testosterone receptor antagonist cyproterone acetate (CPA) interferes with the action of testosterone and considerably reduced the testosterone-induced suppression of nitrite release of leucocytes. The presence of steroid receptors in thymic cells. T-lymphocytes, and synovial macrophages in mammals (Schuurs and Verheul. 1990) provides support for the receptor-mediated action of steroids in cells of the immune system.

Several in vitro experiments also suggest that testosterone is immunosuppressive, inhibiting lymphocyte proliferation, cytokine production, and macrophage activity mammals and birds. Savita and Rai (1998) also reported that estradiol, testosterone and progesterone markedly reduced the nitrite release from LPS-activated macrophages in a dose and time-dependent manner in mice. A parallel finding was described by Brough-Holub and Kraol
(1996). They suggested concentration and exposure time-dependent glucocorticoid regulation of nitric oxide secretion from mammalian alveolar macrophages. Similarly dose and time-dependent sex steroid hormone regulation of TNF release has been demonstrated by Chao et al. (1996) from rat peritoneal macrophages.

In the present study, T-cell mitogens (Con A and PHA) and B-cell mitogen (LPS) induced peripheral blood lymphocyte proliferation was studied. Testosterone treatment in lymphocyte culture did not yield lucid effects. Basal (without any mitogen) as well as mitogen-induced proliferation did not change when different concentrations of testosterone were used. There is no consistency in available reports on lymphocyte proliferation and varied results regarding lymphocyte proliferation have been reported in mammals and birds. Bilbo and Nelson (2001) in hamsters found that testosterone as well as estradiol is stimulatory to lymphocyte proliferation, whereas Macro et al. (2009) in mice reported no significant effect of testosterone on lymphocyte proliferation or cytokine secretion. Anja et al. (2004) reported improved immune responses including lymphocyte proliferation on deprivation of testosterone in rodent model. Deborah et al. (2000) in wild male and female starling birds reported that implant of testosterone significantly reduced humoral immunity in both males and females. Unresponsiveness of splenocytes proliferation towards testosterone was also observed by Klein et al. (1997). al-Asaleq and Homeida (1998) reported in vitro testosterone caused inhibition of macrophage phagocytosis and lymphocyte proliferation in Broiler chickens. All these reports indicate species differences in testosterone effects on immune responses. Present finding reveals testosterone as inhibitory to splenocyte immune response in Natrix piscator.

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