Current Research in Physiology 5 (2022) 355–360

Contents lists available at ScienceDirect

Current Research in Physiology

journal homepage: www.sciencedirect.com/journal/current-research-in-physiology

Evaluation of the sex steroids mediated modulation of leucocyte immune responses in an ophidian *Natrix piscator*

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ARTICLE INFO

Keywords:
Testosterone
Progesterone
Immune responses
Proliferation
Snake

ABSTRACT

The immune-suppressive role of sex steroids in mammals is well documented, but information on other vertebrates is limited. The present study was planned to analyze the effect of testosterone and progesterone in the modulation of immune functions of leucocytes in a reptile, *Natrix piscator*. Reptiles are unique organisms and this study is novel in that it provides an insight into immune-reproductive cross-talk in a reptile. Leucocytes were isolated from peripheral blood, cultured with different concentrations of testosterone and progesterone and different immune parameters like phagocytosis, superoxide production, and nitrite release were assessed. Lymphocytes were isolated and cell-mediated immunity was assessed through proliferation responses utilizing tetrazolium salt. Concentration-dependent suppressive effects of both the steroids on immune responses were observed. A differential suppressive effect of testosterone was also observed when a lymphocyte proliferation assay was studied. Using receptor antagonists such as cyproterone acetate and mifepristone restored the immune responses of cultured cells. It was summarized that gonadal steroids mediate a direct suppressive effect on innate and cell-mediated immune responses of blood immune cells. It was concluded that when gonadal steroids are high in reproductive seasons, the immune functions are suppressed to gain optimum reproductive success.

1. Introduction

Sexual differentiation in immune function is well documented in mammals, where males are generally considered more prone to infections than females (Zuk and McKean, 1996). Sex steroids are deemed responsible for sexual dimorphism in mammals. However, the role of sex steroids in reptilian leucocyte immune alterations is yet to be known. Sex and age-dependent nematode infection in reptiles have been reported (Brown and Symondson, 2014). The endocrine system coordinates with the immune system to gain an optimum response. The differential pattern of innate and adaptive immunity between males and females is attributed to sex steroids such as testosterone, estradiol, progesterone, and glucocorticoids. It is also assumed that the intensity and longevity of immune responses are affected by the specific type of immune cells and their number. Therefore, the influence of sex steroids on the particular immune cell type will determine the overall response of the animal (Bereshchenko et al., 2018). Steroid hormonal control of immune responses affects the differentiation of immune cells into a specialized cell type which decides the kind of immune response manifested (Olsen and Kovacs, 1996).

Gonadal steroids are crucial for reproductive success. However, these steroids are associated with other physiological processes such as immunity. Sex steroids are considered anti-inflammatory (Straub, 2007; Gilliver, 2010). Some experiments have revealed that physiological levels of male sex steroids suppress while female steroids elevate immune responses (Grossman, 1984, 1985). Estradiol exerts its action via the activation of nuclear receptors that regulate gene expression, which is involved in the alteration of immune functions (Hewitt et al., 2016). Estradiol receptors have been identified in various immune cells, including leucocytes, dendrite cells, and macrophages (Klein and Flanagan, 2016). Similarly, androgens generally inhibit immune cells by activating nuclear receptors (Ariga et al., 1989; Chang et al., 1988; Olsen and Kovacs, 1996). Nitric oxide (NO) is an essential toxic defense molecule produced by many immune cells. Savita and Rai (1998) have reported that nitric oxide production declines after dihydrotestosterone administration, but Flynn (1986) found no significant change in cytokine production after *in vivo* treatment with testosterone. The immune system, consisting of various cells, responds to invading pathogens and

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https://doi.org/10.1016/j.crphys.2022.09.003
Received 19 June 2022; Received in revised form 23 August 2022; Accepted 20 September 2022
Available online 21 September 2022

eliminates them to maintain homeostasis. There are reports of differential immune activity in males and females which is attributed to gonadal steroids (Klein and Flanagan, 2016; Ortona et al., 2016). In non-mammals, information on this subject is poor. In reptiles, males exhibit reduced macrophage activity, which is attributed to the presence of androgens (Mondal and Rai, 1999). The reptilian innate immune responses are more diverse and robust (Rios and Zimmerman, 2015; Quesada et al., 2019). Functional characteristics of reptilian leucocytes have been deciphered in snakes (de Carvalho et al., 2017), where the authors have reported the involvement of chemokines in leucocyte migration. Studies on gonadal steroid-mediated alteration of leucocyte immune responses in reptiles are missing. The understanding of immune function across the vertebrate taxa and its modulation by sex steroids will be greatly enhanced by widening the systematic studies among different groups of animals. Studies on reptiles are essential from the comparative point of view as reptiles are significant animals from which evolution occurred and led to birds and mammals. The keelback freshwater ophidian *Natrix piscator* is a seasonal breeder, and in seasonal breeders, reproductive steroids appear to modulate immune surveillance during a particular time of year (Tripathi and Singh, 2014). Understanding the cross-talk between immune-endocrine interactions in non-mammals is vital to reveal the regulation of defense responses. There is a report that the cytotoxic and phagocytic activities of macrophages are altered by sex steroids in the wall lizard *Hemidactylus flaviviridis* (Mondal and Rai, 1999). Looking at the available literature on mammals (Fargallo et al., 2007; Pap et al., 2010) and some studies in lacertilians (Mondal and Rai, 1999), we hypothesized that leucocyte immune responses in *N. piscator* may be modulated by gonadal steroids. To evaluate our hypothesis, we performed this study to assess the role of testosterone and progesterone in the modulation of leucocyte immune responses in *N. piscator*.

2. Materials and methods

2.1. Animals

Six animals were collected from the ponds and ditches situated near the city of Varanasi, India (28^0 18′N; 83^0 01′E). Male snakes were fetched from April to May when they were not active reproductively (Haldar and Pandey, 1989). Animals were kept in a laboratory experiencing natural conditions (temperature 25–35 ^oC; photoperiod 12–13.50 h; humidity 40–45%). Small fishes were available for feeding ad libitum and animals were acclimated for 14 days. All the guidelines recommended by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India, were strictly adhered to.

2.2. Isolation of leucocytes

After acclimation, about 2 ml of blood was immediately isolated from mildly anaesthetized animals through cardiocentesis in heparinized tubes. The leucocytes were separated from whole blood utilizing the method described elsewhere (Keller et al., 2005). In short, the blood was centrifuged (42×*g*) and the leucocytes were segregated from the buffy by slowly rotating the tube. The cells were again centrifuged (200×*g*), the plasma was rejected and 1 ml of culture medium (RPMI 1640) was mixed into the cell pellet. The culture medium was previously supplemented with 10 μl ml^{-1} of 200 mM L-glutamine, 1 μl ml^{-1} of Gentamycin, 10 μl ml^{-1} anti-anti (Gibco) and 5% FBS. The purity and viability of the cells were verified by microscopic examination and the trypan blue exclusion test, respectively. The experiment was continued when viability exceeded 95%. To maintain consistency, all the experiments were carried out by one person.

2.3. Respiratory burst activity and superoxide anion production

Nitroblue tetrazolium (NBT) reduction test is an assessment of the activation of oxidative burst, which has a highly reactive microbicidal effect (Bagasra et al., 1988) in response to antigenic stimulation. In the absence of antigenic stimulation, the NBT reduction test indirectly measures the intracellular hexosemonophosphate shunt activity (Park et al., 1968). Nitroblue tetrazolium salt, purchased from Sigma Chemicals, USA, is a yellow-colored dye that is bioreduced to NBT-diformazan by superoxide. Superoxide anion production was measured by the NBT assay following the method of Berger and Slapnickova (2003). Isolated leucocytes were counted and maintained in a culture medium. Cell suspensions (50 μl) containing 1×10^5 cells were incubated for 4 h in wells of culture plates with equal volumes of different concentrations of testosterone and progesterone (10, 100, and 1000 ng ml^{-1}) in a humidified CO_2 atmosphere at 25^o C. The *in vitro* concentrations of testosterone and progesterone were chosen based on the literature available (Vainikka et al., 2005; Aguila et al., 2013; Tripathi and Singh, 2014) and a pilot experiment. The stock solutions of the steroids were made in dimethyl sulfoxide (DMSO) (1 mg ml^{-1}) and further dilutions were made in RPMI. After incubation, 50 μl of culture medium containing NBT (1 mg ml^{-1}) was added to each well. Plates were again incubated for 2 h, centrifuged, washed and fixed. To dissolve the NBT-diformazan crystals, 2M KOH (120 μl) and DMSO (140 μl) were added and the absorbance was read at 620 nm with the help of a microplate reader.

2.4. Nitrite assay

Nitric oxide is an important effector molecule implicated in the cytotoxic activity of immune cells. Production of NO facilitates cytotoxic and bactericidal activities of immune cells (Shreshtha et al., 2018). The amino acid L-arginine and the enzyme nitric oxide synthase (NOS) are involved in the formation of NO. Because NO is a very unstable molecule, it decomposes quickly to nitrite ($NO2^-$) and nitrate ($NO3^-$), which are known as reactive nitrogen intermediates (RNI) (Jorens et al., 1995). RNI and NO have beneficial effects in immune functions (Delledonne et al., 2003). The method employed by Ding et al. (1988) was used to perform this assay. In the culture plates, leucocytes (1 × 105 cells) were seeded with different concentrations of testosterone and progesterone (10, 100, and 1000 ng ml^{-1}) and incubated at 25 °C for 24 h in an incubator with a 5% CO_2 supply. Separate wells containing leucocytes and sex steroids were added with cyproterone acetate (testosterone receptor antagonist) and mifepristone (progesterone receptor antagonist). After 24 h, the plates were centrifuged and the supernatant was separately mixed with an equal amount of Griess reagent. The Griess reagent was prepared by dissolving 1% sulfanilamide in 3N HCl and 0.1% naphthylenediaminedihydrochloride in distilled water. The absorbance of the pink solution was read at 540 nm with the help of a microplate reader.

2.5. Lymphocyte proliferation assay

Tetrazolium salt, mitogens such as Concanavalin A (Con A), Phytohemagglutinin (PHA), and Lipopolysaccharide (LPS), and other chemicals were procured from Sigma Chemicals, USA. The proliferation of the lymphocyte is a crucial marker of the cell-mediated immune response. Lymphocyte proliferation was assayed through the non-radioactive method developed by Berridge et al. (2005), which involves the use of tetrazolium salt (MTT). Tetrazolium salts are bio-reduced into a colored formazan product by the mitochondrial dehydrogenase of metabolically active cells. The quantity of formazan product as measured by the amount of absorbance at 570 nm light is directly proportional to the number of living cells in culture (Cory et al., 1991). The lymphocyte separation gradient HiSep by the density of 1.077 g ml^{-1} was used to segregate the blood lymphocytes. The blood was layered over the Hisep

and centrifuged (400×*g*) for 30 min. The lymphocyte ring was formed after centrifugation, cautiously separated, washed, and counted and viability was verified. Basal proliferation was studied by seeding 1×10^5 lymphocytes in the wells of the culture plate. Mitogen-activated proliferation was assessed using T-cell mitogens Con A (10 μg ml^{-1}), PHA(10 μg ml^{-1}), and B-cell mitogen LPS (20 μg ml^{-1}). Lymphocytes were cultured with different concentrations of mitogens for 48 h in an incubator having a 5% CO_2 supply. The effects of sex steroids was analyzed by adding different concentrations of testosterone and progesterone (10, 100, and 1000 ng ml^{-1}) into separate wells which had lymphocytes with and without mitogens. After incubation for 48 h, 20 μl of MTT reagent (5 mg ml^{-1}) was added to each well and incubation was further followed for 4 h. Culture plates were then centrifuged and the supernatant was discarded. To dissolve the formazan crystals present inside the cell, 100 μl of DMSO was added and the absorbance of the violet color solution was read at 570 nm with the help of a microplate plate reader.

2.6. Phagocytic assay

Phagocytosis is a conserved innate immune response found in vertebrates. Neutrophil phagocytosis was done utilizing the yeast cells *Saccharomyces cerevisae*. The suspension of the heat-killed yeast cells was prepared, and the suspension was mixed with an equal amount of whole blood. Phagocytosis was allowed for 60 min and a thin smear was drawn on a clean glass slide. The slides were stained with Giemsa stain after drying and examined under a microscope. A total of 100 neutrophils were counted and the percentage of phagocytosis and the phagocytic index were calculated. The phagocytic index was determined by calculating the average number of yeast cells engulfed by a single cell. The percent phagocytosis was calculated by dividing the number of cells showing phagocytosis by 100.

2.7. Statistical analysis

Each experiment was performed three times with six animals to ensure reproducibility. The data is given as mean ± SEM. The data was tested for normality and equality of variance. Data were analyzed by generalized linear model procedure and Analysis of Variance (ANOVA) using SPSS 16.0. Post hoc differences were determined by the Tukey-HSD test. Differences were considered significant when $p < 0.05$. Post hoc comparisons were also made using the Bonferroni corrections to adjust the P values.

3. Results

Testosterone and progesterone significantly inhibited the superoxide production by blood leucocyte (Figs. 1 and 2). *In vitro* testosterone inhibited nitrite release by blood leucocytes, but there was no concentration-dependent suppressive effect. Furthermore, the use of a testosterone receptor antagonist (cyproterone acetate) alleviated the suppressive impact and resulted in nitric oxide production comparable to the control (Fig. 3). We did not find the inhibition of nitrite release by leucocytes when 10 and 100 ng ml^{-1} progesterone were used. The suppressive effect on nitrite release was visible at 1000 ng ml^{-1} progesterone, and the use of mifepristone (progesterone receptor antagonist) resulted in the mitigation of the suppressive effect (Fig. 4). Differential suppressive-effects of steroids were observed on lymphocyte proliferation. Basal and mitogen-activated proliferation was suppressed by testosterone at 100 and 1000 ng ml^{-1} concentrations (Fig. 5). Similar results were obtained when progesterone was mixed in cultures. Basal, as well as mitogen-stimulated proliferation were effectively suppressed at 100 and 1000 ng ml^{-1} concentration (Fig. 6). Testosterone suppressed the neutrophil percentage phagocytosis in a dose-dependent manner where 100 and 1000 ng ml^{-1} concentrations were more effective. The phagocytic index showed phase-wise suppression, where 100 ng ml^{-1} concentration was moderately suppressed while 1000 ng ml^{-1} concentration maximally suppressed the phagocytic index (Fig. 7). Progesterone also suppressed the percentage phagocytic of neutrophils at 100 and 1000 ng ml^{-1} concentrations, but the phagocytic index was suppressed only at 1000 ng ml^{-1} concentration (Fig. 8).

4. Discussion

Many studies have demonstrated the presence of the steroid receptors on immune cells (Nilsson et al., 2001; Kushner et al., 2000; Biswas et al., 2005), which indicates that the immune cell responses are modulated by these hormones. The cells of innate immunity like neutrophils, macrophages, and dendrite cells are modulated by sex steroids (Lai et al., 2012; Klein and Flanagan, 2016). Sex-related disparities in immune status in non-mammals are limited. As far as reptiles are concerned, a few studies demonstrate the modulation of immune functions after *in vivo* treatment of testosterone (Saad et al., 1990). Reduction in the number of lymphocytes in the thymus and spleen has also been reported in *Mauremys caspica* following sex steroid administration (Varas et al., 1991, 1992). In our earlier study, testosterone differentially suppresses the immune functions of splenic macrophages in *N. piscator* (Tripathi and Singh, 2014). In the present study, testosterone significantly suppressed the production of superoxide anion by peripheral

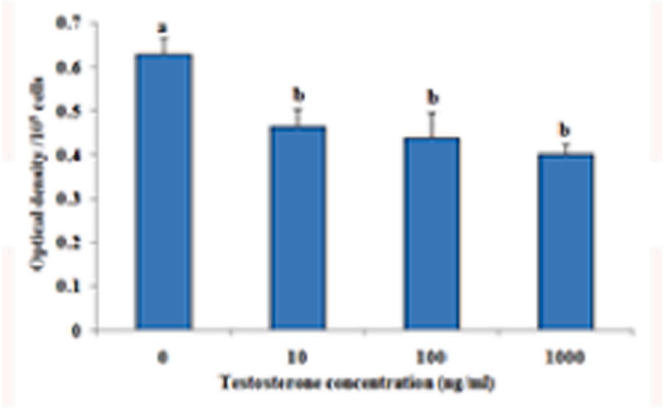

Fig. 1. Effect of different concentrations of testosterone on superoxide production by leucocytes in *Natrix piscator*. The error bars having different letters differ significantly (Newman-Keul's multiple-range test, $F_{3,20} = 7.117$, $P < 0.01$).

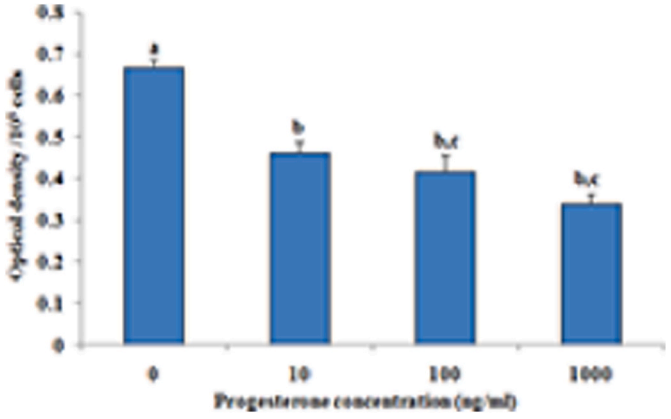

Fig. 2. Effect of different concentrations of progesterone on superoxide production by leucocytes in *Natrix piscator*. The error bars having different letters differ significantly (Newman-Keul's multiple-range test, $F_{3,20} = 68.396$, $P < 0.001$).

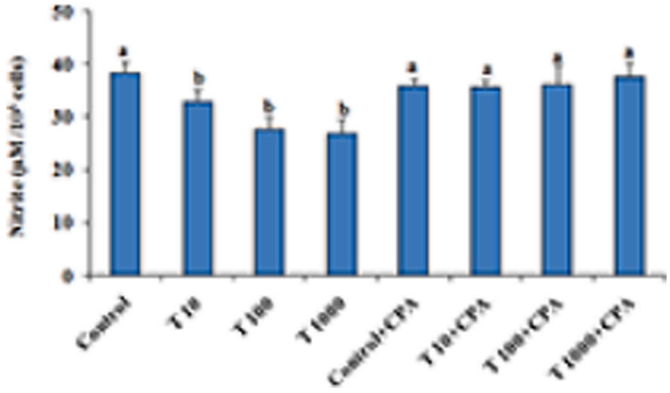

Fig. 3. Nitrite release by leucocytes was suppressed when testosterone was administered in the cultures. Treatment with testosterone receptor antagonist cyproterone acetate (CPA, 100 ng ml^{-1}) resulted in the mitigation of suppressive effect. The error bars having similar letters do not differ significantly (Newman-Keul's multiple-range test, $F_{7,40} = 3.827$, $P < 0.01$).

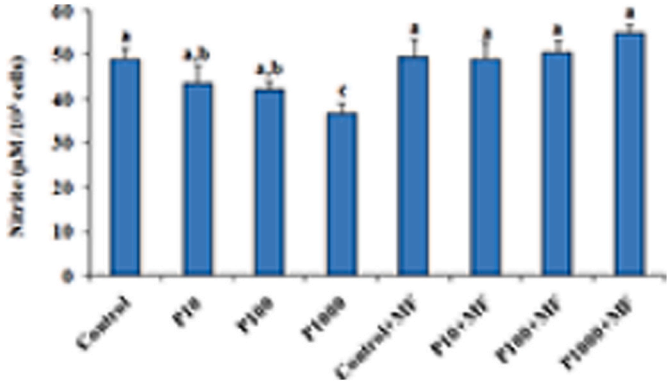

Fig. 4. Progesterone suppresses the nitrite release by leucocytes, however, when progesterone receptor antagonist mifepristone (MF, 100 ng ml^{-1}) was used, the nitrite release was comparable to control cultures. The error bars having similar letters do not differ significantly (Newman-Keul's multiple-range test, $F_{7,40} = 3.729$, $P < 0.01$).

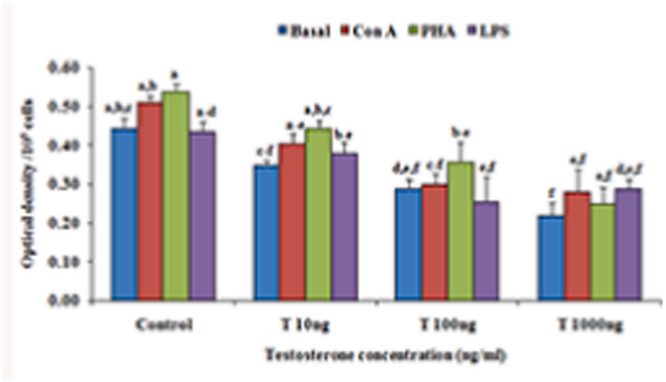

Fig. 5. Effect of different concentrations of testosterone (T 10 to 1000 - Testosterone 10–1000 ng ml^{-1}) on basal and mitogen (ConA: Concanavalin A; PHA: Phytohemagglutinin; and LPS: Lypopolysaccharaide) induced lymphocyte proliferation in *Natrix piscator*. The error bars having different letters differ significantly (Newman-Keul's multiple-range test, $F_{15,80} = 8.262$, $P < 0.001$).

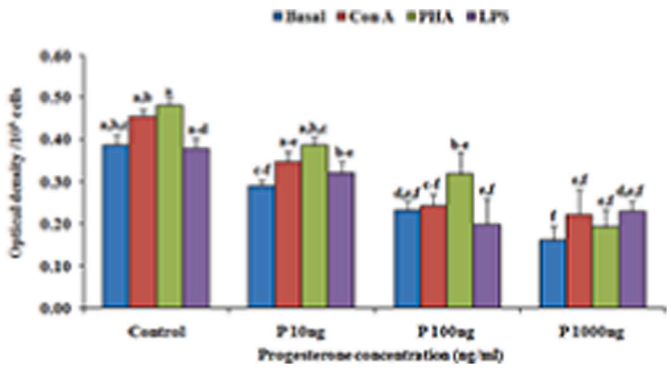

Fig. 6. Effect of different concentrations of progesterone (P 10 to 1000 - Progesterone 10–1000 ng ml^{-1}) on basal and mitogen (ConA: Concanavalin A; PHA: Phytohemagglutinin; and LPS: Lypopolysaccharaide) induced lymphocyte proliferation in *Natrix piscator*. The error bars having different letters differ significantly (Newman-Keul's multiple-range test, $F_{15,80} = 8.262$, $P < 0.001$).

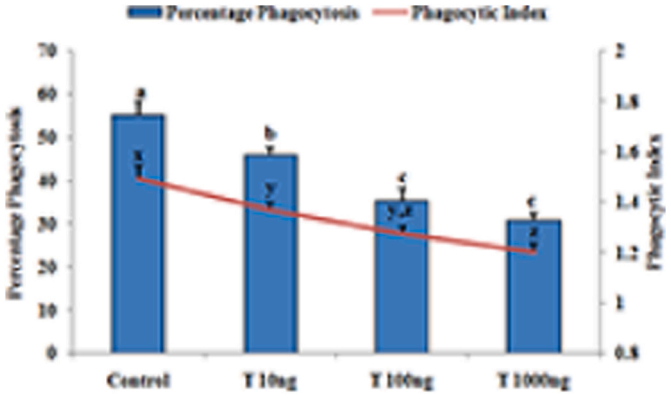

Fig. 7. Effect of different concentrations of testosterone on leucocyte percentage phagocytosis ($F_{3,20} = 30.161$, $P < 0.001$) and phagocytic index ($F_{3,20} = 14.046$, $P < 0.001$) in the fresh water snake, *Natrix piscator*. The error bars having the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

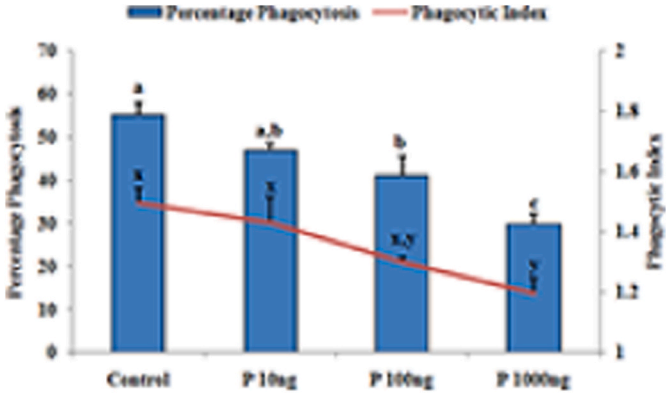

Fig. 8. Effect of different concentrations of progesterone on leucocyte percentage phagocytosis ($F_{3,20} = 25.081$, $P < 0.001$) and phagocytic index ($F_{3,20} = 11.706$, $P < 0.001$) in the fresh water snake, *Natrix piscator*. The error bars having the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

blood leucocytes of *N. piscator*. Direct suppressive effects of testosterone may be possible because immune cells like natural killer cells, dendrite cells, and lymphocytes express gonadal steroid receptors, which lead to alteration in the immune responses (Teilmann et al., 2006). Progesterone-mediated immune alterations can be explained based on results available in mammals as the immune cells treated with progesterone, secrete a lower amount of cytokines when compared to

untreated cells (Butts et al., 2007; Jones et al., 2010). Interestingly, the biphasic suppressive role of gonadal steroid in lizard splenic macrophage superoxide production has previously been reported (Mondal and Rai, 1999).

The production of nitric oxide was reduced in the cultures treated with either steroid. However, the production of nitric oxide by leucocytes was comparable to control cultures when steroid receptor antagonists such as cyproterone acetate and mifepristone were used along with testosterone and progesterone, respectively. The use of receptor antagonists and the notion that sex steroids alter immune functions via binding and activating the intracellular receptors in immune cells go parallel with the observation of Messingham et al. (2001). The results of the present study were also found in the lizard, *Hemidactylus flaviviridis* where authors found a dose and time-dependent reduction in nitrite release by splenic macrophages (Mondal and Rai, 2002). Research from the same laboratory also demonstrated that *in vitro* treatment of sex steroids noticeably reduced the nitrite release from LPS-activated macrophages in a dose and time-dependent manner in mice (Savita and Rai, 1998). Our study has revealed diminished lymphocyte proliferation after *in vitro* treatment of lymphocytes with testosterone and progesterone. Contradicting results were found in mammals as thymic cells were elevated after testosterone treatment (Fitzpatrick et al., 1991; Olsen et al., 2001). In *N. piscator*, T-cell mitogen-activated splenocyte proliferation was suppressed after testosterone treatment (Tripathi and Singh, 2014). In parallel to our previous study, both T and B cell mitogen-stimulated lymphocyte proliferation were suppressed in the present study after treatment of lymphocytes with both the steroids. The postulated mechanism of steroid action in the present study may be understood in light of observations in mammals where increased proliferation and/or decreased apoptosis are the reasons for thymic enlargement in rats (Lai et al., 2012). It has been suggested that sex steroids alter immune functions in two ways. First, the gonadal steroids suppress lymphocyte proliferation; second, lymphocyte responses are altered (Lai et al., 2012). Neutrophil phagocytosis is an important mechanism for combating foreign invaders. A literature survey has revealed that phagocytosis by neutrophils and macrophages is often higher in females than in males (Spitzer, 1999). Sex-specific phagocyte functions of astrocytes have recently been investigated (Castrillo et al., 2020). We have found that testosterone and progesterone suppress the neutrophil phagocyte function in a concentration-dependent manner, where 1000 ng ml^{-1} concentration of both the steroids was effective maximally in phagocytosis inhibition. Literature available suggests that in lizards and snakes, testosterone effectively suppresses the phagocytic functions of macrophages (Mondal and Rai, 1999, 2002; Tripathi and Singh, 2014). The investigation related to immune regulation by gonadal steroids is always fascinating, especially in non-mammals. The energy trade-off is a crucial physiological adaptation in reptiles where the energy is partitioned among different physiological processes (Smith and French, 2017). To cope with the infection in annual breeders, mounting immune responses need considerable energy that could be otherwise allocated to other important processes such as growth, reproduction, etc. Gonadal steroids present a physiological mechanism by reducing the immune responses and thus diverting the energy resources towards reproduction for the successful perpetuation of the species, provided the seasonal immune stressors are low.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Alka Singh: Methodology, Investigation. **Ramesh Singh:** Conceptualization, Supervision. **Manish Kumar Tripathi:** Methodology, Writing – original draft, Reviewing and editing, Investigation, Revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Aguila, S., et al., 2013. Specific and non-overlapping functions of testosterone and 11-ketotestosterone in the regulation of professional phagocyte responses in the teleost fish gilthead seabream. Mol. Immunol. 53, 218–226. https://doi.org/10.1016/j.molimm.2012.08.002.

Ariga, H., Edwards, J., Sullivan, D.A., 1989. Androgen control of autoimmune expression in lacrimal glands of MRL/Mp-lpr/lpr mice. Clin. Immunol. Immunopathol. 53 (3), 499–508. https://doi.org/10.1016/0090-1229(89)90011-1.

Bagasra, O., Howeedy, A., Kajdacsy-Balla, A., 1988. Macrophage function in chronic experimental alcoholism I. modulation of surface receptors and phagocytosis. Immunol. 65, 405–409.

Bereshchenko, O., Bruscoli, S., Riccardi, C., 2018. Glucocorticoids, sex hormones, and immunity. Front. Immunol. 9, 1332. https://doi.org/10.3389/fimmu.2018.01332.

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

Berridge, M.V., Herst, P.M., Tan, A.S., 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. 11, 127–152.

Biswas, D.K., Singh, S., Shi, Q., Pardee, A.B., Iglehart, J.D., 2005. Crossroads of estrogen receptor and NF-kappa B signaling. Sci. STKE 288, pe27. https://doi.org/10.1126/stke.2882005pe27.

Brown, D.S., Symondson, W.O., 2014. Sex and age-biased nematode prevalence in reptiles. Mol. Ecol. 23 (15), 3890–3899. https://doi.org/10.1111/mec.12688.

Butts, C.L., et al., 2007. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. Int. Immunol. 19, 287–296.

Castrillo, A., Garcia-Segura, L., Arevalo, L., 2020. The synthetic steroid tibolone exerts sex-specific regulation of astrocyte phagocytosis under basal conditions and after an inflammatory challenge. J. Neuroinflammation 17 (1). https://doi.org/10.1186/s12974-020-1719-6.

Chang, C.S., Kokontis, J., Liao, S.T., 1988. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 240 (4850), 324–326. https://doi.org/10.1126/science.3353726.

Cory, A.H., Owen, T.C., Barltrop, J.A., Cory, J.G., 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. 3, 207–212.

de Carvalho, M.P.N., Queiroz-Hazarbassanov, N.G.T., de Oliveira Massoco, C., SantAnna, S.S., Lourenco, M.M., Levin, G., Sogayar, M.C., Grego, K.F., Catao-Dias, J. L., 2017. Functional characterization of neotropical snakes peripheral blood leukocytes subsets: linking flow cytometry cell features, microscopy images and serum corticosterone levels. Dev. Comp. Immunol. 74, 144–153. https://doi.org/10.1016/j.dci.2017.04.007.

Delledonne, M., Polverari, A., Murgia, I., 2003. The functions of nitric oxide-mediated signaling and changes in gene expression during the hypersensitive response. Antioxidants Redox Signal. 5 (1), 33–34.

Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediated and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. 141, 2407–2412.

Fargallo, J.A., Martinez-Padilla, J., Toledano-Diaz, A., Santiago-Moreno, J., Davila, J.A., 2007. Sex and testosterone effects on growth, immunity and melanin coloration of nestling *Eurasian kestrels*. J. Anim. Ecol. 76, 201–209.

Fitzpatrick, F., Lepault, F., Homo-Delarche, F., Bach, J.F., Dardenne, M., 1991. Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinol 129, 1382–1390.

Flynn, A., 1986. Expression of Ia and the production of interleukin-1 by peritoneal exudate macrophages activated *in vivo* by steroids. Life Sci. 38, 2455–2460.

Gilliver, S.C., 2010. Sex steroids as inflammatory regulators. J. Steroid Biochem. Mol. Biol. 120 (2–3), 105–115. https://doi.org/10.1016/j.jsbmb.2009.12.015.

Grossman, C.J., 1984. Regulation of the immune system by sex steroids. Endocr. Rev. 15, 435–455.

Grossman, C.J., 1985. Interactions between the gonadal steroids and the immune system. Science 227 (4684), 257–261. https://doi.org/10.1126/science.3871252.

Haldar, C., Pandey, R., 1989. Effect of pinealectomy on testicular cycle of Indian checkered water snake. Natrix piscator. Gen. Comp. Endocrinol. 76, 214–222.

Hewitt, S.C., Winuthayanon, W., Korach, K.S., 2016. What's new in estrogen receptor action in the female reproductive tract. J. Mol. Endocrinol. 56 (2), R55–R71. https://doi.org/10.1530/JME-15-0254.

Jones, L.A., et al., 2010. Differential modulation of TLR3- and TLR4-mediated dendritic cell maturation and function by progesterone. J. Immunol. 185, 4525–4534.

Jorens, P.G., Matthys, K.E., Bult, H., 1995. Modulation of nitric oxide synthase activity in macrophages. Mediat. Inflamm. 4, 75–89.

Keller, J.M., McClellan-Green, P.D., Lee, A.M., Arendt, M.D., 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.

Klein, S.L., Flanagan, K.L., 2016. Sex differences in immune responses. Nat. Rev. Immunol. 16 (10), 626–638. https://doi.org/10.1038/nri.2016.90.

Kushner, P.J., et al., 2000. Estrogen receptor pathways to AP-1. J. Steroid Biochem. Mol. Biol. 74 (5), 311–317. https://doi.org/10.1016/S0960-0760(00)00108-4.

Lai, J.J., Lai, K.P., Zeng, W., Chuang, K.H., Altuwaijri, S., Chang, C., 2012. Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. Am. J. Pathol. 181 (5), 1504–1512. https://doi.org/10.1016/j.ajpath.2012.07.008.

Messingham, K.A., Shirazi, M., Duffner, L.A., Emanuele, M.A., Kovacs, E.J., 2001. Testosterone receptor blockade restores cellular immunity in male mice after burn injury. J. Endocrinol. 169, 299–308.

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

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

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., et al., 2001. Mechanisms of estrogen action. Physiol. Rev. 81 (4), 1535–1565. https://doi.org/10.1152/physrev.2001.81.4.1535.

Olsen, N.J., Kovacs, W.J., 1996. Gonadal steroids and immunity. Endocr. Rev. 17 (4), 369–384. https://doi.org/10.1210/edrv-17-4-369.

Olsen, N.J., Olson, G., Viselli, S.M., Gu, X., Kovacs, W.J., 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. Endocrinol 142, 1278–1283.

Ortona, E., Pierdominici, M., Maselli, A., Veroni, C., Aloisi, F., Shoenfeld, Y., 2016. Sex-based differences in autoimmune diseases. Ann. Ist. Super Sanita 52 (2), 205–212. https://doi.org/10.4415/ANN_16_02_12.

Pap, P.L., Czirjak, G.A., Vagasi, C.I., Barta, Z., Hasselquist, D., 2010. Sexual dimorphism in immune function changes during the annual cycle in house sparrows. Naturwissenschaften 97, 891–901.

Park, B.H., Fikrig, S.M., Smithwick, E.M., 1968. Infection and nitroblue-tetrazolium reduction by neutrophils: a diagnostic aid. Lancet 2, 532–534.

Quesada, V., Freitas-Rodríguez, S., Miller, J., Peerez-Silva, J.G., Jiang, Z.F., Tapia, W., Santiago-Fernaandez, O., Campos-Iglesias, D., Kuderna, L.F.K., Quinzin, M., et al., 2019. Giant tortoise genomes provide insights into longevity and age-related disease. Nat. Ecol. Evol. 3, 87–95. https://doi.org/10.1038/s41559-018-0733-x.

Rios, F.M., Zimmerman, L.M., 2015. Immunology of Reptiles. eLS, pp. 1–7. https://doi.org/10.1002/9780470015902.a0026260.

Saad, A.H., Khalek, N.A., Ridi, R., 1990. Blood testosterone level: a season-dependent factor regulating immune reactivity in lizards. Immunobiology 180, 184–194.

Savita, Rai, U., 1998. Sex steroid hormones modulate the activation of murine peritoneal macrophages: receptor mediated modulation. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 119, 199–204.

Shreshtha, S., Sharma, P., Kumar, P., Sharma, R., Singh, S.P., 2018. Nitric oxide: it's role in immunity. J. Clin. Diagn. Res. 12 (7), BE01–BE05. https://doi.org/10.7860/JCDR/2018/31817.11764.

Smith, G.D., French, S.S., 2017. Physiological trade-offs in lizards: costs for individuals and populations. Integr. Comp. Biol. 57 (2), 344–351. https://doi.org/10.1093/icb/icx062.

Spitzer, J.A., 1999. Gender differences in some host defense mechanisms. Lupus 8, 380–383.

Straub, R.H., 2007. The complex role of estrogens in inflammation. Endocr. Rev. 28 (5), 521–574. https://doi.org/10.1210/er.2007-0001.

Teilmann, S.C., Clement, C.A., Thorup, J., Byskov, A.G., Christensen, S.T., 2006. Expression and localization of the progesterone receptor in mouse and human reproductive organs. J. Endocrinol. 191 (3), 525–535. https://doi.org/10.1677/joe.1.06565.

Tripathi, M.K., Singh, R., 2014. Differential suppressive effects of testosterone on immune function in fresh water snake, *Natrix piscator*: an *in vitro* study. PLoS One 9 (8), e104431. https://doi.org/10.1371/journal.pone.0104431.

Vainikka, A., et al., 2005. Effects of testosterone and b-glucan on immune function in tench. J. Fish. Biol. 66, 348–361. https://doi.org/10.1111/j.0022-1112.2005.00598.x.

Varas, A., Saad, A.H., Torroba, M., Zapata, A., 1991. Structural changes in the thymus gland of turtle following testosterone treatment. Thymus 17, 129–132.

Varas, A., Torroba, M., Zapata, A.G., 1992. Changes in the thymus and spleen of the turtle *Mauremis caspica* after testosterone injection: a morphometric study. Dev. Comp. Immunol. 16, 165–174.

Zuk, M., McKean, K.A., 1996. Sex differences in parasite infections: patterns and processes. Int. J. Parasitol. 26, 1009–1024.