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Existence of NOS/NO system in testis and its relation with reproductive activity in the common carp, cyprinus carpio

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Abstract

Nitric oxide has been known as a modulator of reproductive system. Its signaling plays an important role in spermatogenesis and steroidogenesis. Demonstrations of NO/NOS system in testis are restricted to the catfish only. Since fishes exhibits wide range of reproductive mode and mechanism under specific ecological niches, there is a need to expand such study in number of other fishes so that a generalized as well as evolutionary aspect of NOS/NO system could be traced. Therefore, attempts were made to investigate the expressions of different forms of NOS in testis along with NO content in testis and serum. Relation of testicular NO with circulating and testicular testosterone was also observed.

Keywords: Nitric oxide synthase; immunohistochemistry; nitric oxide; testosterone

Introduction

Nitric oxide (NO) is a gaseous versatile signaling molecule produced from L-arginine. Its synthesis is catalyzed by one of the three isoforms of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). All three isoforms of NOS are demonstrated in several tissues of vertebrates including testis in mammals (Stuehr et al., 1991; Burnett et al., 1992; Schmidt et al., 1992; Wolff and Datto, 1992; Nakane et al., 1993; Weiner et al., 1994 Tatsumi et al., 1997; Weissman et al., 2005), though the predominant form differs from cell to cell and species to species (Roselli et al., 1998; Lee and Cheng, 2004). Initially it was believed that NO reaches its intracellular targets by freely diffusing through the cell membranes.

The survey of the existing literature suggest that the important role of NO in gonadal activities. Nitric oxide neurons are present in many hypothalamic nuclei, including sites involved in regulation of GnRHsecretion and thereby gonadotropins.NO is reported to directly modulate the androgen production by Leydig cells through autocrine (Del Puntaet al., 1996; nee Pathak and Lal, 2008) and/or paracrine pathways (Weissman et al., 2005; nee Pathak and Lal, 2010). However, mechanism involved in are equivocal. It inhibits testicular testosterone production *in vivo* (Gaytan et al., 1997) as well as *in vitro* (Del Punta et al., 1996; Pomerantz and Pitelka,1998; Kostic et al., 1998; Sharma et al., 1998). On the contrary, Valentiet al. (1998) have demonstratedthat NO stimulates testosterone production in rat testis.They, subsequently, reported that NO exerts inhibitory effect as well on Leydig cell steroidogenesis depending on its concentration (Valentiet al., 1999).

Nevertheless, studies on regulation of testicular steroidogenesis by NO/NOS system are confined to mammals and less report are available in ectothermic vertebrates, particularly in fishes, though different is forms of NOS have been demonstrated in variety of tissues in cyprinid, cichlid and salmonid fishes (see review Agnisola and Pellegrino, 2007;Pelster,2007). iNOS and nNOS has been demonstrated by cloning and sequencing of full-length genomic DNA from gills of rainbow trout and through their immunohistochemica llocalization in the nerve fibers and epithelial cells in the gill of killfish, *Fundulusheteroclitus* (Hyndmanet al., 2006). Hyndmanet al. (2006) have also cloned and characterized a putative NOS homologue from the brain of the killfish. They also demonstrated the expression of nNOS mRNA in the gill, stomach, kidney, opercular epithelium, intestine and heart through relative quantitative RT-PCR.

However, demonstration of NO/NOS system in testis is restricted to the catfish, *Clariasbatrachus* only (nee Pathak and Lal, 2008, 2010). Since fishes exhibit wide range of reproductive modes, there is a need to expand such study in number of other fishes so that a generalized as well as evolutionary aspect of NOS/NO system could be traced. The present study was, therefore, undertaken to demonstrate the presence of isoforms of NOS in testis of the common carp, *Cyprinus corpio* with simultaneous analyses of NO content and testosterone. Circulating NO and testosterone were also measured.

Materials and methods

Fish

Freshwater carp, *C.carpio* was collected from ponds in suburbs of Varanasi (28˚08’ N; 83˚01’ E) India, in November (reproductively immature) and February (reproductively mature) months.

Sample collection

Fish were weighed and sacrificed to collect blood separately in microfuged tubes by caudal puncture. Blood cells were allowed to settle for 3h at room temperature (~25˚C) and then tubes were centrifuged at 5000rpm for 15min at 4^0C. Serum was isolated and kept frozen at -20^0C for further analyses. Testes were quickly removed, weighed and washed in 0.6% saline, blotted and kept frozen at -20˚C till analysis. Some pieces of testes were also fixed in Bouin’s fluid. Gonadosomatic index of the catfish was calculated using following formula:

GSI=Gonadal weight / body weigh X 100

Histology

The fixed testicular pieces were processed next-day through ascending series of alcohol for dehydration, cleared in xylene and blocks were prepared in paraffin. Then after, 6µm thick transverse sections of the testes were cut and processed for haematoxylin/ eosin staining.

Immuno his to chemical localization of nNOS, iNOS, eNOS like molecules in the testis

Paraffin sections of testes collected from immature and mature *C. carpio* were de-parafinized, hydrated gradually and finally washed with PBS thrice (0.01M, pH 7.3). The endogenous peroxidase activity in the testicular sections was removed by treatment with (0.4% v/v) hydrogen peroxide in methanol. The sections were then processed for the immunohistochemical localization of NOS like molecules employing HRP conjugated antibody methods (nee Pathak and Lal, 2008). Briefly, the testis sections were first incubated with 5% normal goat serum for 2h at room temperature. Thereafter, it was drained, washed and incubated with rabbit anti-iNOS (Sigma-Aldrich, USA) diluted to 1:750, anti-nNOS polyclonal antibody (Sigma-Aldrich, USA) and anti-eNOS at 1:100 dilutions (Sigma-Aldrich, USA), separately, for 12h at room temperature. Parallel to it, non-immune rabbit serum was also employed in place of primary antiserum to determine the level of nonspecific immunoreactivity and served as control. Slides were then washed with PBS thrice, (15min each). Thereafter, incubated with HRP conjugated secondary antibody (Bangalore Genei, India) at 1:100 dilution for 1h at room temperature, washed with PBS, and subjected to chromogen substrate (DAB-20mg/ml Tris buffer-0.1M, pH-7.6) for 10min in dark at room temperature. The reaction was then stopped by rinsing the slides in PBS three times for 10min and processed for routine permanent mounting in DPX. Slides were viewed under Carl Zeiss Axioscope II microscope (Carl Zeiss, Germany) having Digi Cool High Resolution Camera and images captured under bright field.

Total nitrate estimation

Total nitrate estimation was done according to the procedure of (Katrina et al, 2001) with minor modifications. Briefly, testicular homogenate (10% w/v) in phosphate buffer was prepared and centrifuged at 7000rpm for 15min at 4^0C. Standard was made with potassium nitrate (100µM) of varying concentrations of 20, 40, 60, 80 and 100µM. Respectively protein precipitation was done using ethanol and diluted three times by adding 200µl ethanol to 100µl homogenate. The mixture was kept on ice for 10-15min and again centrifuged. In a 96 well microtitreplate, 100µl sample supernatant, 100µl vanadium trichloride and 100µl freshly prepared Greiss reagent was added and color was allowed to develop for 45min in dark and then O.D. was read at 545nm.

Testosterone assay

A highly sensitive and specific commercial ELISA kit (Dia.Metra, Italy-DK0002) was used for analysis of testosterone concentration in the testicular homogenate and serum. The protocol supplied with kit was followed for testosterone assay. For the routine determination of testosterone, 25µl of serum, testes homogenate and testosterone standards were taken in quadruplicate wells of the microtitre plate as per the protocol. Thereafter, 100µl of testosterone conjugate was added to each well, mixed and incubated at 37^0C for 1h. Following incubation, content of the each well was flicked properly and washed twice (5min each) with 300µl distilled-water.

Thereafter, 100µl of TMB-substrate was added to each well and incubated at room temperature for 15min in dark. Reaction was then stopped by adding 100µl of stop solution. The amount of enzyme complex was analyzed by measuring optical density at 450nm (Thermo Lab system Multiscan EX-355) against blank. Blank wells were incubated only with TMB substrate.

Results

Morphological changes in the testis

In the immature testis, the diameters of seminiferous tubules were smaller (Fig.1) and interstitium was poorly developed. The germinal epithelium was consisted of spermatogonial stem cells and Sertoli cells, and the lumen was filled with primary spermatogonia in cysts. The mature testis showed well-developed interstitium with large number of interstitial cells. Seminiferous tubule was full of cysts containing advanced stages of germ cells and spermatozoa. GSI of the mature carp was higher than the immature carp (Fig.2).

Localization of NOS like molecules in the testis

Immunoreaction for nNOS like molecule was detected in both the testicular compartments (seminiferous tubules and interstitium) of the reproductively immature and mature carp testis. A moderate immunoreaction for nNOS was more in germ cells in immature testis, which decreased in the mature testis germ cells(Fig. 3), while nNOS expressed more in the interstitial cells in mature testis as compared to the immature test is. A distinct immunoprecipitation of iNOS like molecule was more in the less differentiated germ cells of the immature testis than that of the advanced germ cells in mature carp testis. However, iNOS expression in the interstititum was relatively more in the mature testis than immature testis (Fig. 4). The immunoreaction for eNOS was more in the both the compartments of mature testis as compared to immature testis (Fig. 5).

Testosterone and nitric oxide concentration in the testis and serum:

Circulating and testicular testosterone levels were significantly high in the immature carp than the mature carp (Fig. 6), while NO was low in immature testes than mature testes. The pattern of variation in NO Level in the serum was almost similar to that of the testicular variation (Fig. 7).

Discussion:

This study reports two important aspects of carp testis physiology: 1) the testes express all the three isoforms of NOS (nNOS, eNOS and iNOS) which vary with reproductive status, and 2) testicular NO seems to be involved in testosterone production. The testicular development in *C. carpio* commences with mitotic proliferation of primary and secondary spermatogonia in the late immature testis. The spermatogenic process accelerates later and attains peak (meiotic division of spermatogonia to yield large number of secondary spermatocytes and spermatids) in

the mature testis. Interstitial cells also proliferate and become hypertrophied gradually from the immature to mature period, corresponding with highest steroidogenic activity. (Chaves-Pozo et al. 2005) have also reported similar cyclic changes in morphology and physiology of both the testicular compartments: seminiferous tubules and interstitium in fishes.

Moreover, the present study reports expressions of NOS like molecules in germ cells as well as interstitial cells. Moderate immunoreaction for nNOS like molecule was detected in germ cells in immature stage which decreased in mature testis. A moderate immunoprecipitation of nNOS like molecule was also observed in interstitial cells in mature testis. Distinct immunoreactivity for iNOS like molecule was detected in the less differentiated germ cells than the advanced germ cells in mature testis. Immunoreaction for eNOS in the interstitium as well as seminiferous tubules was more in mature testes than immature testes. The varying degree of expression of NOS like molecules in immature and mature testes suggest that fish testis has good potential of generating NO during both the reproductively inactive and active periods, and the cellular source of NO are numerous. Relatively low immunoreaction of nNOS and eNOS as compared to the iNOS may be attributed to low expression of nNOS and eNOS.

The existence of NOS isoforms have been reported in interstitial cells like Leydig, endothelial, peritubular and macrophage cells, as well as different cell types in seminiferous tubules such as Sertoli cells, spermatogonia, spermatocytes and spermatids in human, rodents and pig (Ambrosino et al., 2003; see review Lee and Cheng, 2004) and Asian catfish (nee Pathak and Lal, 2008, 2010).

In mammals, NO has been reported to play vital role in regulation of steroidogenesis (see review Rosselli et al., 1995; Del Punta et al., 1996). It has also been postulated that expressions of iNOS and eNOS activate programmed cell death by generating NO (Lee and Cheng, 2004). NO is also shown to stimulate germ cell differentiation. In light of these reports, therefore, the NOS like molecules may be involved in germ cells differentiation in *C. carpio*.

Amongst many suggested functions of NO in testis, role in testicular testosterone production is thoroughly studied in mammals but reports are contradictory and equivocal. Testosterone production remains always under tight control of stimulatory and inhibitory factors, and a delicate balance between these opposing factors determines the pattern of testosterone production. It is documented that NO has very high affinity for binding to iron-containing enzymes. For example, NO binds to haem prosthetic group of guanylate cyclase and increases intracellular cGMP (Ignarro, 1990; Dixit and Parvizi, 2001).

In the present study, a negative relation between testicular testosterone and NO levels indicates that NO may be involved in the testosterone production in *C.carpio* perhaps by binding to cytochrome P450 enzyme. As the testicular and circulating

testosterone levels were high in the immature testes, NO levels were low and vice-versa in the mature testis. Nevertheless, the possibilities of other mode of NO action on down regulation of testosterone production in *C. carpio* cannot be ruled out.

Thus, the present study demonstrates the existence of nNOS, eNOS and iNOS like molecules in varied cell types in fish testis. The expression of these NOSs also vary with the reproductive status of the carp. Testicular NO appears to play role in regulation of spermatogenesis and steroidogenesis.

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29. Legend to the Figures:

30. **Fig. 1.**Representative images of hematoxylin-eosin stained transverse sections of immature and mature testes of *C. Carpio.* (A) Immature testis (B) Mature testis. Seminiferous tubule (ST), Interstitium (IST)

31. **Fig. 2.**Gonadosomatic index (GSI) of immature and mature *C. carpio.*

32. **Fig. 3.** Representative images of transverse section of immature and mature testis of *C. Carpio* localizing nNOS like molecule immunohistochemically. Interstitium (IST), seminiferous tubule (ST), Germ cell (GC).

33. **Fig. 4.** Representative images of transverse section of immature and mature testis of *C. Carpio* localizing iNOS like molecule immunohistochemically. Interstitium (IST), seminiferous tuble (ST), Germ cell (GC).

34. **Fig. 5.** Representative images of transverse section of immature and mature testis of *C. Carpio* localizing eNOS like molecule immunohistochemically. Interstitium (IST), seminiferous tuble (ST), Germ cell (GC).

35. **Fig. 6.** Levels of testosterone in serum and testis of immature and mature *C. Carpio.* Data represented are mean ± SEM (n=5).

36. **Fig. 7.** Levels of nitrate/nitrite in serum and testis of immature and mature *C. Carpio.* Data represented are mean ± SEM (n=5).

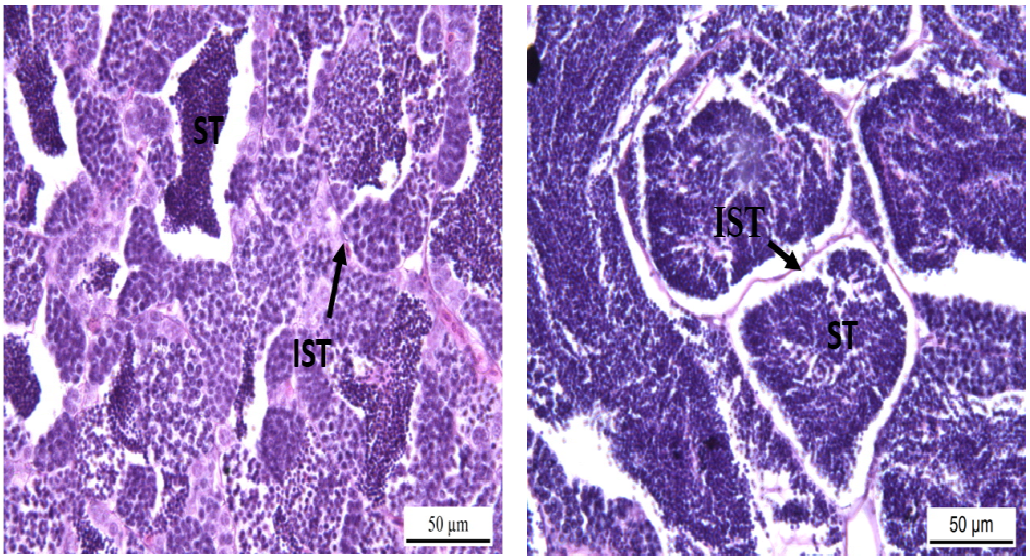

Fig. 1. Represenattive images of hematoxylin-eosin stained transverse sections of immature and mature testes of *C. Carpio* . (A) Immature testis (B) Mature testis. Seminiferous tubule (ST), Interstitium (IST)

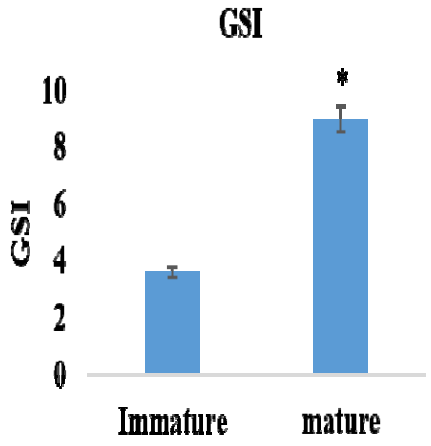

Fig. 2. Gonadosomatic index (GSI) of immature and mature *C. carpio.*

a. Immunolocalization of nNOS:

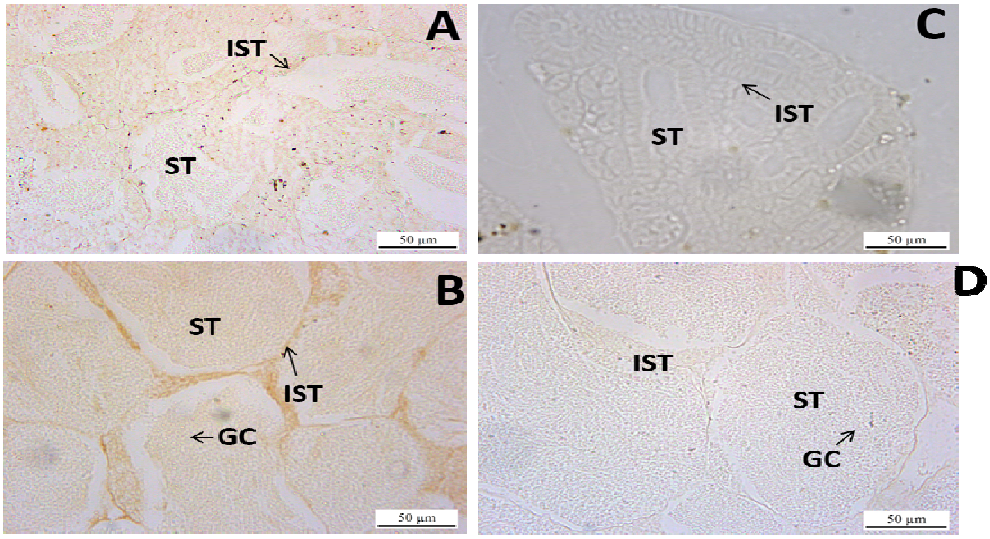

Fig. 3. Representative images of transverse section of immature and mature testis of *C. Carpio* localizing nNOS like molecule immunohistochemically. Interstitium (IST), Seminiferous tuble (ST), Germ cell (GC).

b. Immunolocalisation of i-NOS:

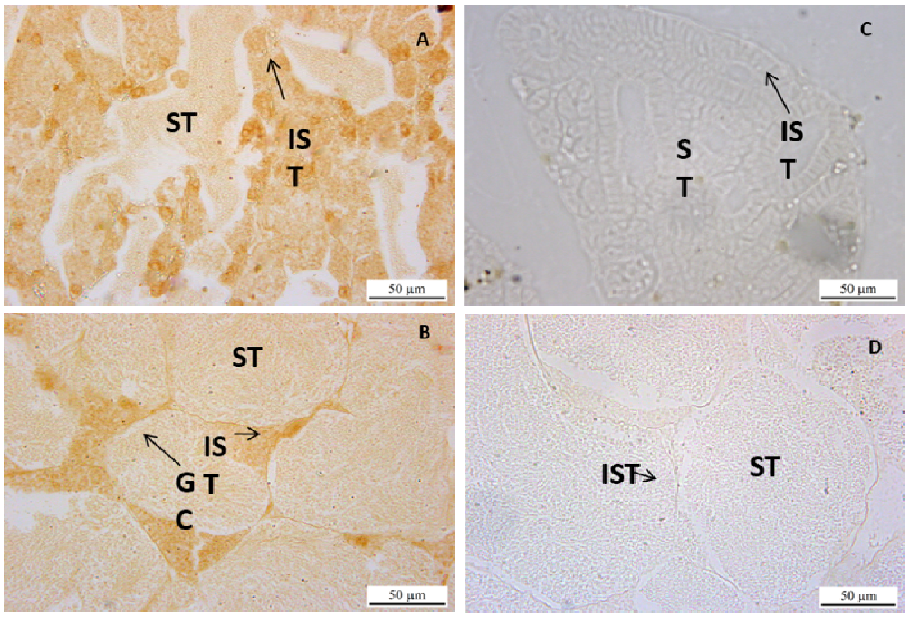

Fig. 4. Representative images of transverse section of immature and mature testis of *C. Carpio* localizing iNOS like molecule immunohistochemically. Interstitium (IST), Seminiferous tuble (ST), Germ cell (GC).

c. Immunolocalisation of e-NOS:

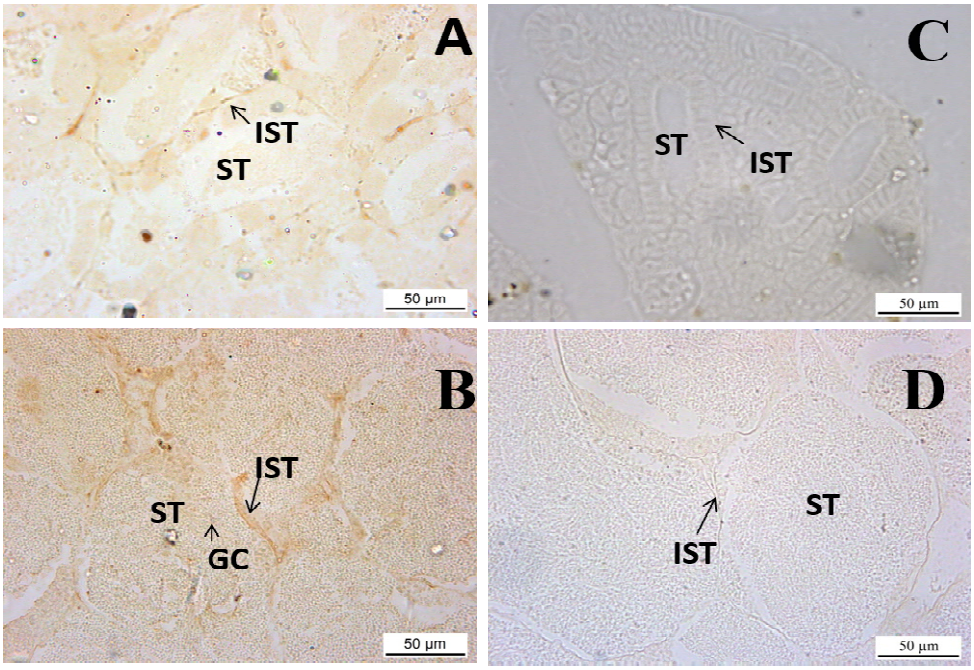

Fig. 5. Representative images of transverse section of immature and mature testis of *C. Carpio* localizing eNOS like molecule immunohistochemically. Interstitium (IST), Seminiferous tuble (ST), Germ cell (GC).

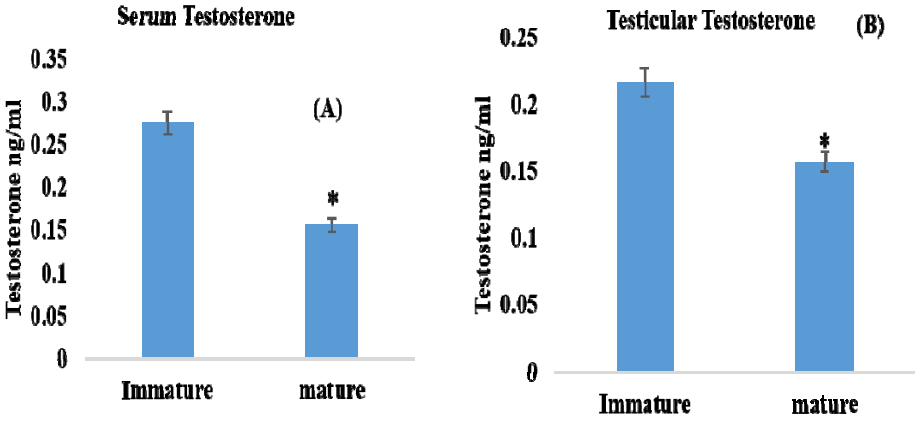

Fig. 6. Levels of testosterone in serum and testis of immature and mature *C. Carpio*. Data represented are mean ± SEM (n=5).

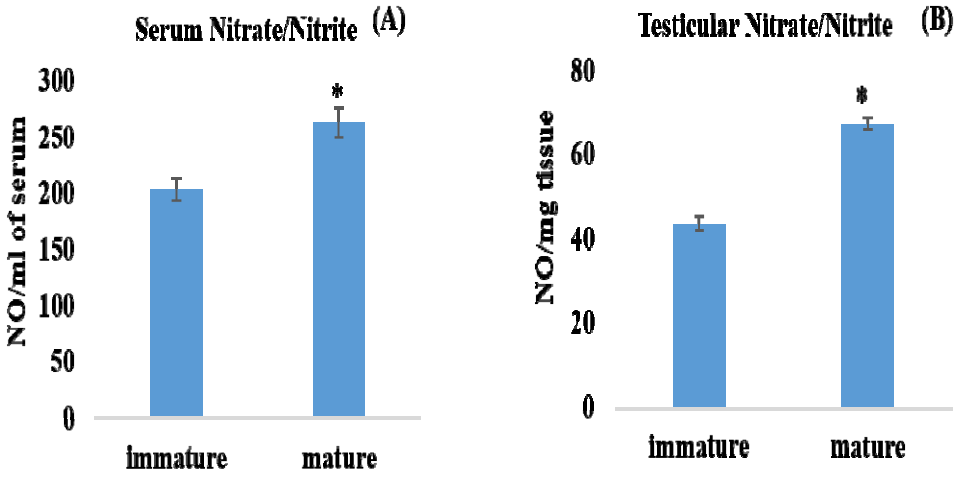

Fig. 7. Levels of Nitrate/Nitrite in serum and testis of immature and mature *C. Carpio*. Data represented are mean ± SEM (n=5).