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Correlation of Nitric oxide and testicular activity in laboratory mouse, *Mus musculus*

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Abstract: This study helps us to understand the interrelationship between the signaling molecule nitric oxide (NO) and testicular activity. In the first part of the experiment, NO activity (nitrite and nitrate concentration; in the plasma and testes and NOS (nitric oxide synthase) expression; in testis) was analyzed in sexually immature and mature laboratory male mouse, Musmusculus. In the second experiment immature mice were administered with NOS inhibitor N-nitro-l-arginine methyl ester (L-NAME, 1 mg/100 g body weight) and mature with NO donor sodium nitroprusside (SNP, 0.25 mg/100 g body weight) intraperitoneally for 7 days. In the third experiment, both immature and mature mice were administered with testosterone propionate (TP) for 7 days intramuscularly. Results indicate increased NO level and intense expression of ir-NOS (immunoreactive NOS) in sexually immature mice compared to mature. Although NOS inhibitor has no effect on plasma testosterone level of immature mice; NO donor has suppressed testosterone in mature mice. Further, testosterone injection decreased NO level in both the age groups of mice. It is concluded that NO and gonadal activity exhibit inverse relationship not only in control/ basal condition but experimental induction of increased NO level may suppress gonadal activity while exogenous testosterone may suppress NO activity. These findings not only suggest the inverse NO-gonadal relationships but also indicate that modulation of NO activity may affect gonadal activity and vice versa under different physiological / experimental conditions.

Keywords: Nitric oxide, L-NAME, SNP, ir- NOS, Testosterone propionate

I. INTRODUCTION

Nitric oxide (NO) a well-known messenger in different physiological processes is also reported to play a major role in the control of gonadal functions [12]. NO plays different role in different vertebrate species as far as reproduction is concerned. In catfishes, NO inhibits testosterone production in the testis [27]. In avian species Japanese quail, gonadal activity exhibits parallel relationship with NO activity [8, 20]. In mammalian species it was found that the NO plays an important role in both normal and inflammatory condition [5], it activates the release of LHRH [25] and can alter the expression of estrogen receptors and aromatase [1]. In general, studies indicate a positive correlation between NO and gonadal function [4, 8, 20, 21, 28, 30, 32], although few contradictory finding are also reported [6, 7, 27]. However, experimental in vivo studies to support correlation of these two systems are scanty.

Nitric oxide has been shown to exert negative effects on steroidogenesis [10], possibly through a direct action on steroid-secreting cells rather than via an effect on local ovarian blood flow [9]. The impairment of steroid production by NO has been demonstrated in different species and in different conditions [rat (9); human (34, 40); porcine (16, 23, 24, 29); bovine (2 , 3)]. The negative effect of NO on steroid production has been demonstrated by treating cultured granulosa-luteal cells with SNP, a NO donor, or with L-NAME, a NOS inhibitor, which markedly decrease or stimulate, respectively, both estradiol and progesterone release. This effect seems to be cGMP independent [human (34, 40); bovine, (2)], even though different conclusions have been drawn in other species [swine (16); rat (17)]. The negative effect of NO on both basal- and gonadotropin-stimulated estradiol production in the rat may be, at least in part, exerted through an inhibition of androstenedione secretion [13]; in addition, the cytochrome P450 aromatase, responsible for estradiol production, has been shown to be possibly inhibited [40]. This inhibition may be exerted through a reduction of aromatase messenger RNA (mRNA) levels and/or of enzyme effect human, [18, 37].

Inspite of a number of studies reporting NO influence on reproduction effects of testosterone on NO activity has not been tested to complete the picture of NO-gonadal relationship. Hence present study was undertaken with the three specific aims i) to understand NO- gonadal relationship in different physiological conditions i.e in sexually immature and mature condition ii) to monitor if modulation of NO level can influence gonadal function and vice versa iii) to investigate the effect of exogenous administration of testosterone on NO level.

These simple but composite study not only supports inverse correlation of two systems in basal condition but provides experimental evidences that modulation of one factor can influence other and vice versa.

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II. MATERIAL AND METHODS:

A. *Animals*

Laboratory male mice, Mus musculus (order rodentia, family muridae) used in the present study were obtained from the mice colony of our laboratory maintained under photoperiodic regimen of LD 12:12. Animals were supplied with food (standard rodents food pellets supplied by PashuAahar Kendra, Varanasi) and tap water ad libitum. The experiment was conducted in accordance with Institutional practice and within the framework of the revised animals (Scientific procedures) act of 2002 of the Government of India on Animal welfare.

B. *Experimental Design*

In the first experiment three weeks old sexually immature and ten weeks old sexually mature (post pubertal) male mice (n=5) were weighed, anaesthetized and sacrificed by decapitation. Except that, another group of fifty weeks (old) male mice were also sacrificed for the same process. Blood was collected in heparinised tubes, centrifuged and plasma was stored in -200C until assayed for total nitrite and nitrate concentration. The testes and seminal vesicle were dissected out, weighed and testes were processed for the estimation of total nitrite and nitrate (NOX) concentration.

In the second experiment, 3 weeks old sexually immature mice were weighed and administered i.p with L NAME (1 mg/100 g body weight) and 10 weeks old sexually mature mice with SNP (0.25 mg/100 g body weight). These injections were prepared in normal saline, continued for 1 week and thereafter mice were sacrificed and processed as mentioned above.

In the third experiment, 3 weeks old sexually immature and 10 weeks old sexually mature male mice were weighed and divided into two groups (n=6). Control group received normal saline and the mice of experimental group were administrated with testosterone propionate (aquaviron, 0.1mg/g body weight) for 7 days. All the injections were given intramuscular in 0.1 ml of solution. Twenty four hours after the last injection the mice were weighed, sacrificed by decapitation and processed as mentioned earlier.

C. *Gonado Somatic Index (GSI)*

GSI is used for measuring the gonadal status/ weight of animals in relation to the body weight and is expressed as GSI (%) by using formula; Gonad Weight / Body Weight x 100.

D. *Total nitrite and nitrate concentration*

NO is a reactive free radical and it is generally oxidized in NOx (nitrite/ nitrate). The quantification of NO formation can be done by measuring the nitrite/ nitrate (NO2-/NO3-) level, which are stable breakdown products of NO which is a short lived molecule. Total nitrite and nitrate concentration were measured in plasma and testis by the method of Sastry et al. [35].

Briefly, for plasma nitrite and nitrate concentration, blood was collected in a heparinised tube and centrifuged at 3000xg for 30 min to separate plasma. 10 % tissue homogenate (w/v, testis) was prepared in 0.01 M phosphate buffer pH 7.4. To 100 µl of each sample (plasma and testis homogenate) or standard was added 400 µl of carbonate buffer followed by a small amount (~0.15) of activated copper-cadmium alloy filings and incubated at room temperature with thorough shaking. At the time of use, the alloy was washed with carbonate buffer and dried on a filter paper. The reaction was stopped by the addition of 100 µl of 0.35M NaOH followed by 120 Mm ZnSO4 solution under vortex and allowed to stand for 10 min.Tubes were then centrifuged at 8000xg for 10 min. 100 µl of aliquots of clear supernatant were transferred into the wells of a microplate (in quadriplicate) and Griess reagent (50µl of 1% sulphanilamide prepared in 2.5% orthophosphoric acid and 50µl of 0.1% N-naphthylethylenediamine prepared in distilled H20) was added to it. After 10 min, the absorbance was read at 545 nm in an ELISA reader (ECIL, India). A standard graph was plotted against different concentrations (0, 20, 40, 60, 80 and 100 µM) of KNO3.

E. *Testosterone assay*

A radioimmunoassay (RIA) of plasma testosterone was performed using a commercial RIA kit (Immuno-tech, Marseille, France) according to the manufacturer's instructions. Sensitivity of the assay was 0.025 ng/ml with extremely low cross reactivities against related molecules (5α- dihydrotestosterone, Δ 4-androstenedione, etc.). Precision for intra-assay and inter-assay variation was 14.8% and 15%, respectively.

F. *Immunohistochemistry*

Immunostainingof polyclonal NOS (nitric oxide synthase), having immunogenecity to the C-terminus of mouse iNOS and nNOS was performed on transverse sections of the testis (6 µm) of sexually immature and mature mice. Detection of ir-NOS (immunoreactive NOS) was done by using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) as described by Srivastava et al. [38] with some modifications.

Hematoxylin-Eosin staining of testis was also performed in sexually immature and mature groups. Slides were viewed and images were captured under Carl Zeiss Axioskop 2 Plus microscope.

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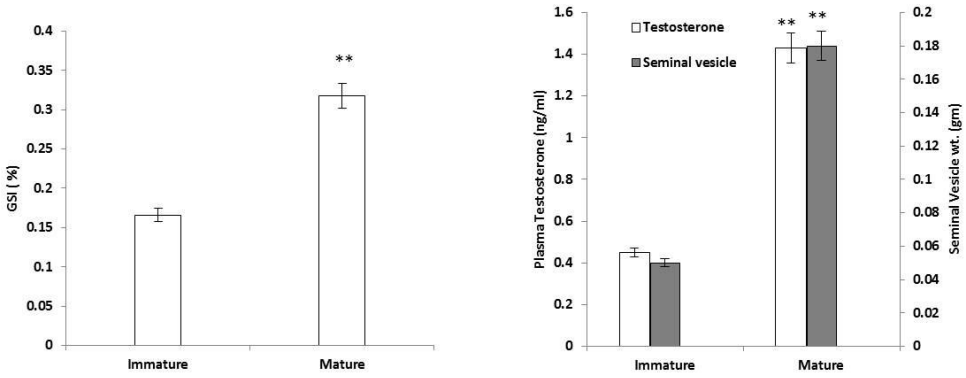
G. *Statistical Analysis*

In experiment I, data was analysed by non-parametric Mann- Whitney test to compare the two groups. The student "t" test was performed to compare between the control and experimental group. Data was expressed as mean ± SEM. Significance was tested at the level of $p < 0.05$.

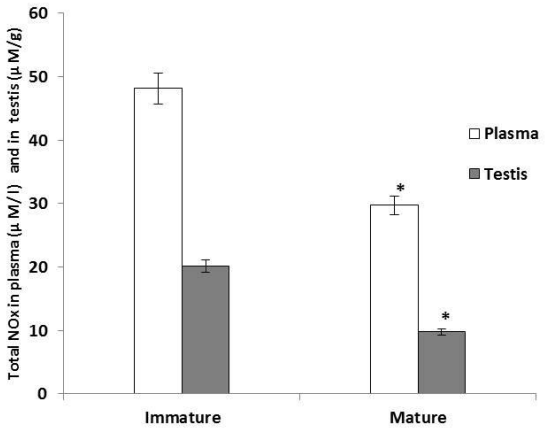
III. RESULTS

A. *Experiment I:*

GSI % , seminal vesicles weight and plasma testosterone level was significantly low in the immature mice compared to mature one (Fig.1.a.). However, total nitrite and nitrate (NOX) concentration in the plasma and testis was significantly high in sexually immature mice compared to the sexually mature male mice (Fig.1.b.). Plasma NOx concentration was also found high in old age mice compare to mature. Expression of ir-NOS was found high in the inner part of seminiferous tubules of sexually immature mice compare to mature (Fig.2).



1.a.



1.b.

Fig.1.a. Upper panel GSI, weight of seminal vesicle and plasma testosterone level in sexually immature and mature mice. Lower panel (1.b.) Total nitrite- nitrateconcentration (Nitric oxide level) in sexually immature and mature mice. Data are mean ± S.E., * $p<0.05$,** $p<0.01$ significance of difference from sexually immature mice.

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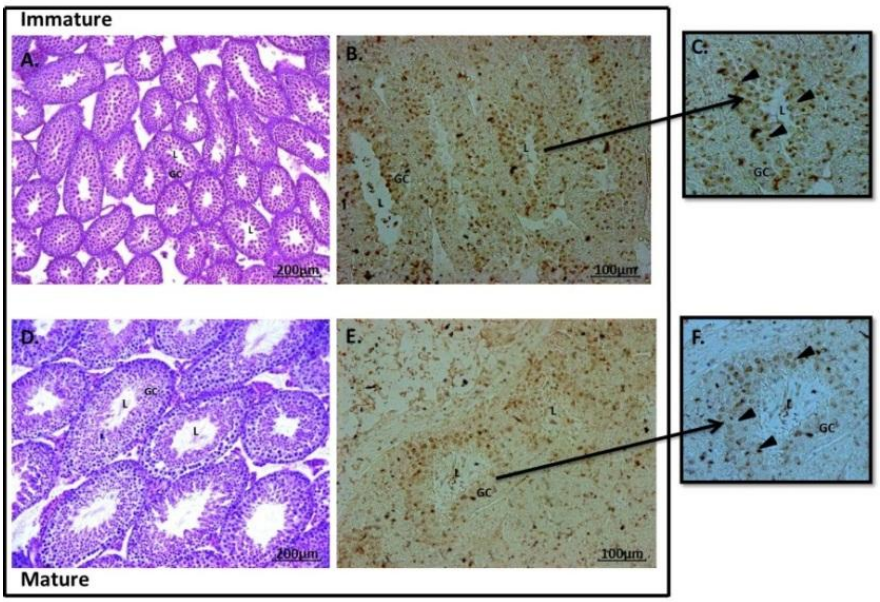


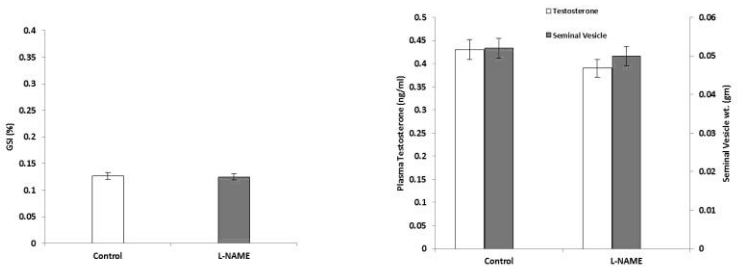
Fig.2.

Fig.2. T.S of testis of mice stained with Haematoxylin Eosin (immature;A and mature; D) and localization of ir-NOS (immature; B,C and mature; E,F). Here GC is germ cell and L is the lumen.

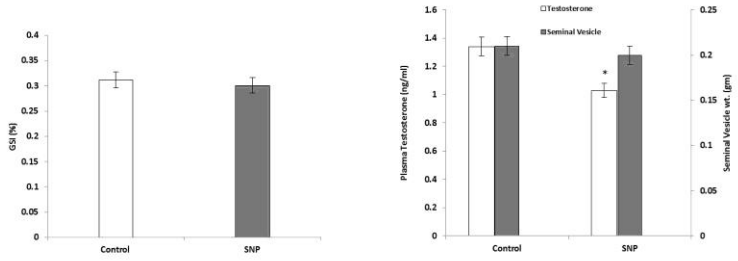
Note the intense immunoreactivity of ir-NOS (marked by the arrow head) in the seminiferous tubules (germ cells) of sexually immature mice (C) compared to mature (F).

B. *Experiment II:*

No change was observed in the GSI %, seminal vesicle weight and plasma testosterone level of L-NAME treated mice immature compare to control (Fig. 3.a.) while administration of SNP in mature mice induced a decrease in plasma testosterone level but GSI % and seminal vesicle weight remained unaffected (Fig. 3.b.).



3.a.



3.b.

Fig.3.a. (Upper panel) Effect of L- NAME administration on GSI, weight of seminal vesicle and plasma testosterone in sexually immature mice.Lower panel

(3.b.) Effect of SNP administration on GSI %, weight of seminal vesicle and testosterone level in sexually mature mice. Data are mean ± S.E.,

* $p<0.05$, significance of difference from control.

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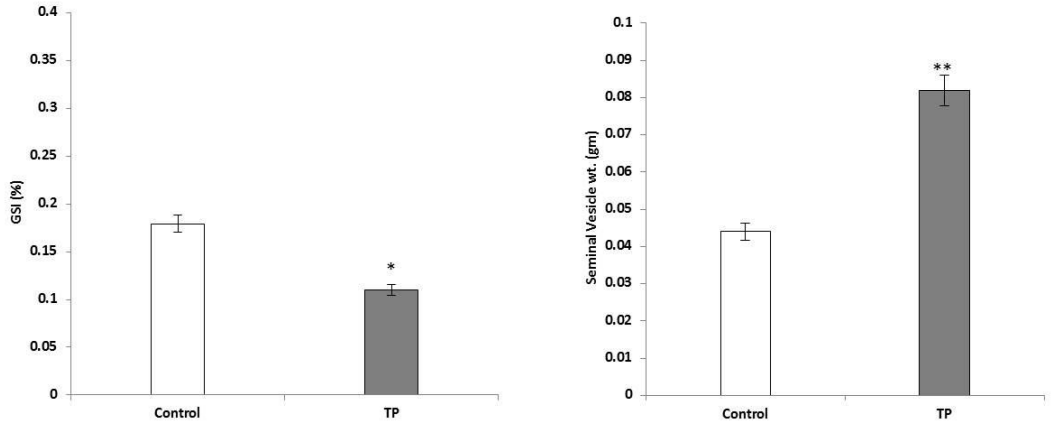
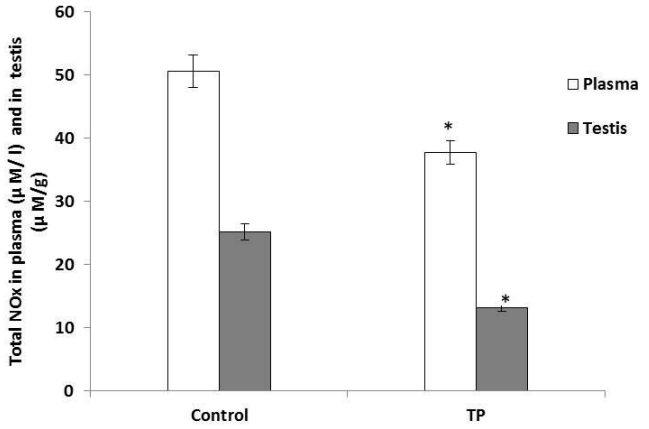
C. *Experiment III.*

Testosterone treatment increased the body weight of sexually immature mice but there was no significant change in the testicular weight resulting in decreased GSI % compared to saline treated control (Fig.4a.). However the seminal vesicle size and weight increased in the testosterone propionate treated group (TP) compared to control (Fig. 4.b.). On the other hand, nitrite/ nitrate concentration decreased in the testis and plasma of testosterone treated mice compared to that of control (Fig. 6). Secondly mature mice treated with testosterone also showed an increase in body weight, decrease in GSI % and increase in seminal vesicle size and weight (Fig. 5.a.) NOx significantly decreased in the testis of TP treated group (Fig. 5.b.) as in sexually immature mice.



4.a.



4.b.

Fig.4.a. Effect of testosterone propionate (TP) administration on the GSI and size and weight of seminal vesicle in sexually immature mice. Lower panel

(4.b.)Effect of testosterone propionate (TP) administration on NO level in sexually immature mice. Data are mean± S.E., * $p<0.05$, ** $p<0.01$

significance of difference from control.

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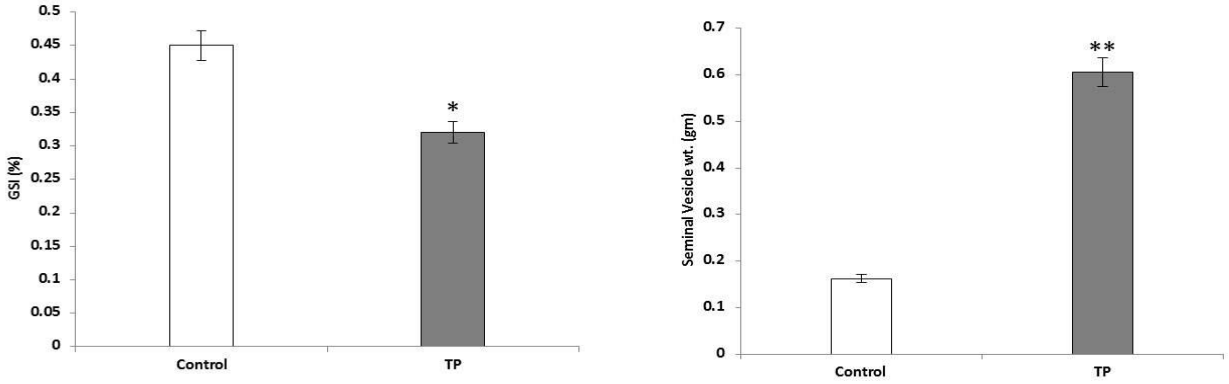
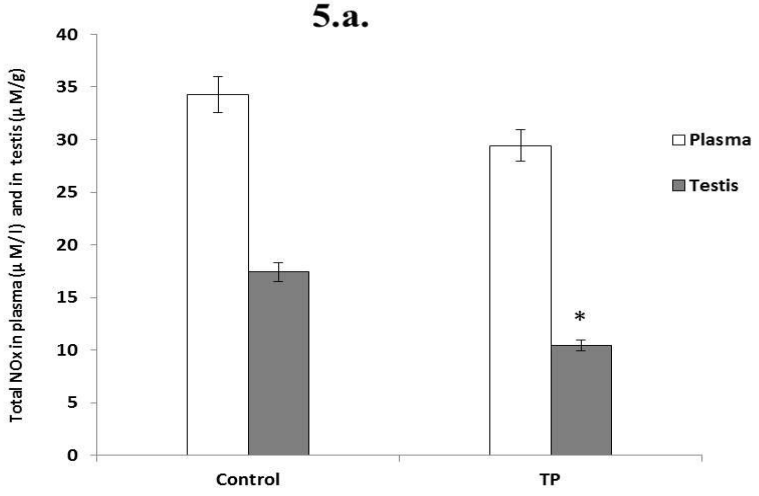


5.a.



5.b.

Fig.5.a. (Upper panel) Effect of testosterone propionate (TP) administration on the GSI and size and weight of seminal vesicle weight in sexually mature

mice. Lower panel (5.b.)Effect of testosterone propionate (TP) administration on NO level in sexually mature mice. Data are mean± S.E.,

* $p<0.05$,** $p< 0.01$,significance of difference from control.

IV. DISCUSSION

This preliminary study demonstrates that in mouse, NO (as judged by NO3/NO2 concentration in the testis and plasma and ir-NOS activity in testis) and gonadal activity (as judged by sex organs weight and plasma testosterone level) not only exhibit an inverse relation (Fig.6) but the modulation of NO activity affects the activity of gonad and vice versa. In control conditions, sexually immature and mature mice exhibit increased and decreased level of NO respectively. On the other hand, administration of NO donor drug (SNP) lowers the testosterone level in mature mice within a week and administration of testosterone decreases NO level in both sexually immature and mature mice. These experimental findings strongly support the inverse NO-gonadal relation in mice. In that way, we suggest that continuous increase in NO level during post pubertal age may be one of the cause of reducing fertility in aging males.

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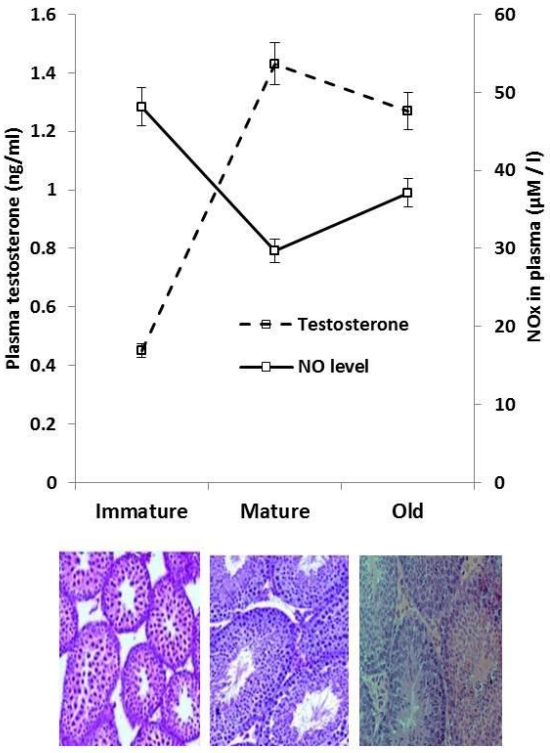

Fig.6

Fig.6. Testosterone, NO level and testicular histology of different age group mice exhibiting inverse correlation. (Immature; 3weeks old, mature; 10 weeks old,
old; 50 weeks old).

Regulation of HPG axis is brought about by a complex network which involves hypothalamic, intrahypophyseal and peripheral signals. Growing evidences suggest that nitric oxide may act as a novel transmitter in hypothalamus [2, 26, 31], pituitary [7] and gonads [13, 14]. Neuronal NOS has also been demonstrated within folliculostellate cells and gonadotrophs of the anterior pituitary (6, 22, 25] suggesting the involvement of NO in reproduction. In sharp contrast to stimulatory role of NO on reproduction Ceccatelli et al [6] reported that NO donor molecules inhibited GnRH stimulated LH release from the dispersed anterior pituitary cells. The inhibition of NOS facilitates LH release from rat pituitaries in vivo and increased LHRH stimulates LH release from the pituitary in vitro [7]. It is also reported that the castration increases and androgen decrease NOS activity in rat brain [36]. Testosterone is also reported to have inhibitory effect on hypothalamic NOS activity in prepubertal rats through GnRH[33].

Based on the available literature it also appears that NO exerts a biphasic effect on testosterone secretion, which is stimulatory at low and inhibitory at high concentrations [39]. The stimulatory effect of NO is mediated by cGMP, the classic second messenger for NO action onleydig cells cultured in vitro. The effect of L-NAME and SNP on in vitro testosterone secretion was analyzed and found that NAME (10 mM) did not affect basal testosterone production, but inhibited the human chorionic gonadotropin stimulated testosterone secretion [28]. In the last few years, however, several studies have reported that testicular nonandrogenic factors are also involved in the regulation of LH secretion. Intratesticularly treatment with SNP is reported to induce a decrease in serum and interstitial fluid testosterone concentrations, without significant changes in LH concentrations after 60 or 120 min [15]. Based on the available literature NO is reported to influence GnRH neurons as well as steroidogenic cells, suggesting its influence on reproduction either through HPG axis or directly on gonad. Although present study clearly provides experimental evidences of inverse NO-gonadal relationship and suggests that modulation of one system can alter other.The mechanism of such action yet to be investigated.

Similar to NO-gonadal relationship (both parallel as well as inverse) NO also exhibits contradictory effects in the regulation of apoptosis; both pro and anti-apoptotic [11]. Variation in the level of NO in different tissues is possible due to presence of different isoforms of NOS (e, n and i-nos) found in different amount and in different physiological conditions.

V. CONCLUSION

From this present preliminary study it is concluded that NO and gonadal activity maintain negative correlation under control/ basal condition. Further, these findings not only suggest the inverse NO-gonadal relationship but also indicate that experimental modulation of NO activity may affect gonadal activity and vice versa under different physiological conditions.

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