Original Article

N^G-nitro-L-arginine Methyl Ester Protects Against Hormonal Imbalances Associated with Nicotine Administration in Male Rats

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Abstract

Background: The administration of nicotine is associated with altered hormonal imbalances and increased serum and testicular nitric oxide (NO) level. **Aim:** This study sought to investigate the effects of NO inhibition with N^G-nitro-L-arginine methyl ester (L-NAME) on altered hormonal imbalance in adult male albinorats. **Materials and Methods:** Rats were administered with 0.5 mg/kg body weight (BW) and 1.0 mg/kg BW nicotine and were treated with L-NAME in the drinking water or drinking water alone for 30 days. Serum was analyzed for testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin using radioimmunoassay. **Results:** Nicotine administration significantly decreased ($P < 0.05$) testosterone in the low and high dose treated groups and FSH in the high dose treated group when compared with the control group. There was a significant increase ($P < 0.05$) in mean LH and prolactin level in the high dose treated group when compared with the control. Concomitant treatment with nicotine and L-NAME produced significant increases in testosterone and FSH, and a decrease in prolactin in 1.0 mg/kg BW. L-NAME alone did not lead to a significant increase in testosterone when compared with control. **Conclusion:** These data demonstrate that the suppressive effects of nicotine on testosterone level of the adult male rat can be prevented by NOS blockade with L-NAME. It appears that these beneficial effects are mediated primarily within the gonad; however, the involvement of the pituitary cannot be totally ruled out.

Keywords: Nicotine, Nitric oxide synthase inhibitor, Rats, Testosterone

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Introduction

Several studies both on humans and animals have associated nicotine use through cigarette smoking with infertility in males and females through a mechanism associated with altered hormonal imbalance in previous studies, implicating nicotine has the major constituent causing these imbalances.[1-3] There are some evidence that male infertility is associated with poor sperm characteristics and decrease in the testicular weight.[4] Much of the early histological analysis of the testis suggests that nicotine concentration at high doses resulted in testicular degeneration and disorganization in the cytoarchitecture resulting in the detrimental effects of nicotine on germ cells, peritubular structures, and Sertoli cells.[5,6]

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Nitric oxide (NO), a ubiquitous diffusible molecule and short-lived free radical is produced by cells in different organs and regulates several physiological functions such as regulation of cell growth, apoptosis, neurotransmission, smooth muscle cell tone, platelet adhesion and aggregation, and injury as well as infection-induced immune reactions.[7] Since these processes are also linked with normal functioning of reproductive processes in male and females, it is suggested that NO plays a vital role in reproduction. Studies have reported

that NO at normal levels is beneficial to the optimal functioning of the reproductive system, while hyper levels can be harmful.[8] Similarly, overproduction of NO in other biological systems is associated with neuronal disorders[9] as well as immune, cardiovascular,[10] and several inflammatory diseases.[11] NO at physiological levels may play an important role in homeostasis, while NO produced under oxidative stress conditions may evoke specific tissue injury.[11] Evidence suggests that NO synthase (NOS) blockade has been used to prevent suppression of the hypothalamic-pituitary-gonadal axisin pubertal male rat.[12] The association between NO and hormone regulation has been earlier documented. Studies have shown neuronal expression of NOS mRNA in the diagonal band of Broca, an area of the hypothalamus heavily populated with luteinizing hormone releasing hormone (LHRH) neurons.[13] Studies have also shown that NO facilitates LHRH secretion[14-16] and NOS has been localized to specific cells in the anterior pituitary, but it is still unclear whether it has a facilitatory or inhibitory effect on gonadotropin release. At the testicular level studies, NO is present in the Sertoli and Leydig cells and has an inhibitory effect.[17-20]

NOS inhibition has been investigated in several biological systems. Caldwell *et al.*,[21] suggested that inhibition of NOS activity decreases lipid peroxidation in the gerbil model of cerebral ischemia and more recently the role of NOS inhibition as an antioxidant has been investigated.[22]

In spite of the growing knowledge of the effects of nicotine on male reproduction through hormonal imbalance and the adverse effect of increased NO levels associated with nicotine administration, there is no information on the effects of NOS inhibition. The aim of this study was to assess the ameliorative effect of NOS blockade through N^G-nitro-L-arginine methyl ester (L-NAME) on nicotine-induced altered hormonal profile in adult male albino rats.

Materials and Methods

Animals and treatments

Experiments were performed on 48 male albino Wistarrats, aged 10-12 weeks and weighing between 180 and 200 g. They wereobtained from the Animal House, Central Animal House, College of Medicine, University of Ibadan, bred and housed in the animal house of the Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan. The animals were allowed to acclimatize for 2 weeks before the commencement of the study. Rats were randomly allotted into groups for various experiments in a well-ventilated room maintained at an ambient temperature (22 ± 2°C), on a 12-h light-dark cycle with free access to tap water and rat chow (Product number BA2012R86, Ladoke Feeds and Flourmills Limited, Ibadan, Nigeria). All animal research procedures were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with The Guide for the Care and Use of Laboratory Animals. The male animals in the six groups were treated orally by gastric intubation at 12:00 h daily for 30 days and they included the control group that received 0.2 ml/kg normal saline (vehicle), 0.5 mg/kg nicotine-treated group, 1.0 mg/kg nicotine-treated group, 50 mg/kg L-NAME, 0.5 mg/kg nicotine alongside with 50 mg/kg L-NAME, and 1.0 mg/kg nicotine alongside with 50mg/kg L-NAME. The duration (30 days) and dose of 0.5 and 1.0 mg/kg for nicotine was selected based on a study by Oyeyipo *et al.*,[3] where nicotine significantly decreased serum level of male reproductive hormones in albino rats, while L-NAME doses selection was based on a study by Arnal *et al.*,[23] where it inhibited NO formation.

Drug preparation

Nicotine preparation

Nicotine hydrogen tartrate (95% nicotine; BDH Chemicals Ltd, Poole, England) was used in the study. The nicotine dosage freshly prepared in normal saline for each group of animals was delivered at 0.5 and 1.0 mg/kg per body weight (BW). The working solutions were stored in foil-wrapped glass bottle at 4°C for no longer than 10 days to ensure potency as previously reported.[6]

NO synthesis inhibition

N^G-nitro-L-arginine methyl ester (L-NAME; Sigma Chemicals, St Louis, MO, USA), a NOS inhibitor, was administered at a dose calculated to provide 50 mg/kg/day to rats. This was administered in light-proof bottles for a period of 30days. It was used to determine the role of NO synthesis in nicotine-induced infertility.

Blood sample collection

On the 31st day of the experiment, animals were humanely killed by euthanasia (intraperitoneal injection of 160 mg/kg pentobarbital) and exsanguination. Blood (2 ml) was collected from each animal via the retroorbital sinus with 70 µl heparinized capillary tube put into plain sample bottle for hormonal analysis.

Hormonal assay

Blood samples were spun at 2,500 rpm for 10 min in a table top centrifuge. The serum samples obtained were analyzed to determine the concentration of testosterone, LH, FSH, and prolactin. In order to minimize the effect of diurnal fluctuation, all samples were obtained in baseline conditions between 8:00 and 9:30 am.

The analysis was carried via the tube-based enzyme immunoassay (EIA) method. The protocol used for the hormone was according to the method described for the kit (kit product codes IM101, IM102, IM103, and IM109 for LH, FSH, prolactin, and testosterone, respectively; Immunometrics Limited, UK) and meet the World Health Organization (WHO) standards in research program for human reproduction.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by the Duncan test for pair-wise between group comparisons. *P*-values of less than 0.05 were accepted as significant. Analysis of data was done using the Statistical Package for Social Sciences (SPSS) software for windows (SPSS Inc, Chicago, IL, USA).

Results

Effect of nicotine and L-NAME on BW

The BW changes of animals were comparable throughout the experiment period as shown in Table 1, indicating thatthe procedures and treatments used were without overt systemic toxicological effects.

Effect of nicotine and L-NAME on serum level of testosterone

The mean serum testosterone level in rats that received 0.5 mg/kg BW and those that received 1.0 mg/kg BW of nicotine for 30 days was significantly decreased ($P < 0.05$) when compared with the control group. This decrease was dose-related. However, 0.5 mg/kg BW + L-NAME and 1.0 mg/kg BW + L-NAME showed no significant change ($P > 0.05$) when compared with the control. There was also an insignificant increase ($P > 0.05$) in serum level of testosterone in the L-NAME only treated group when compared with the control group as shown in Figure 1.

Effect of nicotine and L-NAME on serum level of LH

The mean serum LH level in rats that received 1.0 mg/kg BW (high dose) of nicotine for 30 days was significantly increased ($P < 0.05$) when compared with the control group. However, 0.5 mg/kg treated, 0.5 mg/kg BW + L-NAME and 1.0 mg/kg BW + L-NAME showed no change when compared with the control.

There was a significant increase ($P < 0.05$) in serum level of LH in the L-NAME treated group when compared with the control group as shown in Figure 2.

Effect of nicotine and L-NAME on serum level of follicle stimulating hormone (FSH)

Results showed that the mean serum FSH level in rats that received 1.0 mg/kg BW of nicotine for 30 days was significantly decreased ($P < 0.05$) when compared with the control group. However, other experimental groups had comparable values with the control as shown in Figure 3.

Effect of nicotine and L-NAME on serum level of prolactin hormone

Results showed that the mean serum prolactin level in rats that received 1.0 mg/kg BW of nicotine for 30 days was significantly increased ($P < 0.05$) when compared with the control group. However, 0.5mg/kg BW, 0.5 mg/kg BW + L-NAME and 1.0 mg/kg BW + L-NAME showed no significant change ($P > 0.05$).

There was a significant decrease ($P < 0.05$) in the mean serum level of prolactin in the L-NAME treated group when compared with the control group as shown in Figure 4.

Discussion

The harmful effects of nicotine on the male rodent reproductive axis in adults are well-recognized. We report herein, for the first time in adult animals, data testing the hypothesis that blockade of NOS, the enzyme that catalyzes the biosynthesis of NO from L-arginine, can prevent the well-known fall in testosterone induced by nicotine. L-NAME used in this study is a potent broad spectrum inhibitor of all the NOS isoforms and have been used in several studies both *in vivo* and *in vitro* when investigating the effects of NO in various

Table 1: Body weight changes of experimental rats treated with nicotine and L-NAME

Dose	Before treatment (g)	Week 1(g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Control	195.22±3.12	208.53±5.10	213.87±4.33	225.00±4.33	235.44±4.26
L-NAME (50 mg/kg BW)	193.43±4.61	210.34±4.87	216.32±5.01	227.08±6.12	235.24±3.44
0.5 mg/kg BW	187.32±3.52	200.43±5.01	210.30±4.16	214.93±5.67	218.25±5.11
1.0 mg/kg BW	190.63±5.44	200.30±4.21	209.48±4.95	213.42±5.01	219.56±5.32
0.5 mg/kg+L-NAME	195.11±5.31	208.13±3.36	214.01±3.99	218.47±5.29	230.13±4.17
1.0 mg/kg+L-NAME	200.91±4.45	212.90±4.32	218.15±4.32	225.96±4.99	233.12±3.43

Values are expressed as means ± SEM of 8 rats per group, L-NAME = N^G-nitro-L-arginine methyl ester, SEM = Standard error of mean, BW = Body weight

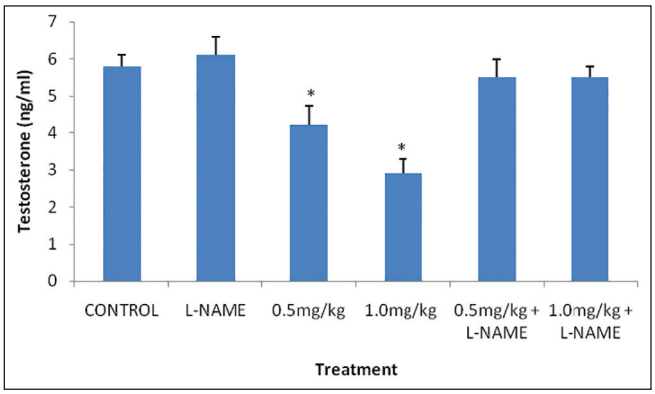

Figure 1: Serum testosterone level in male rats treated with nicotine and L-NAME. Values are expressed as mean ± SEM of 8 rats. * = $P < 0.05$ vs control

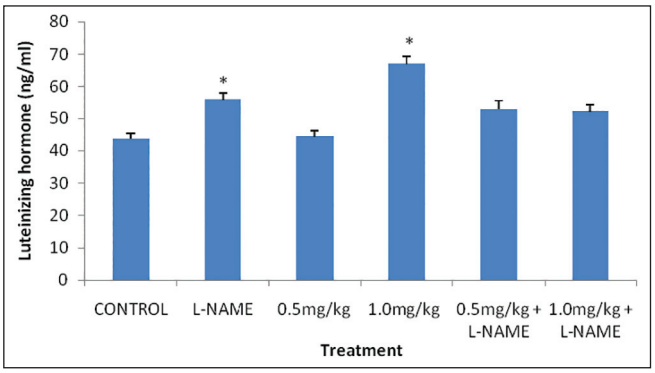

Figure 2: Serum luteinizing hormone level in male rats treated with nicotine and L-NAME. Values are expressed as mean ± SEM of 8 rats. * = $P < 0.05$ vs control

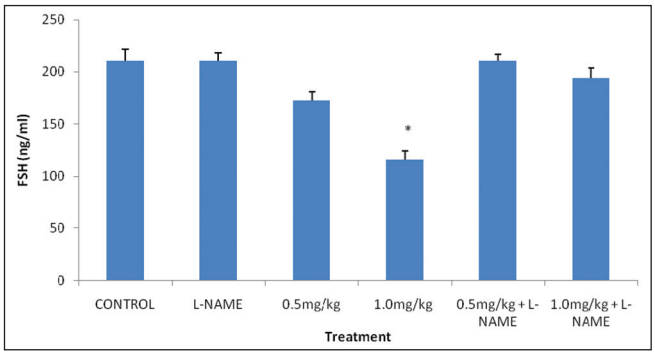

Figure 3: Serum follicle stimulating hormone level in male rats treated with nicotine and L-NAME. Values are expressed as mean ± SEM of 8 rats. * = $P < 0.05$ vs control

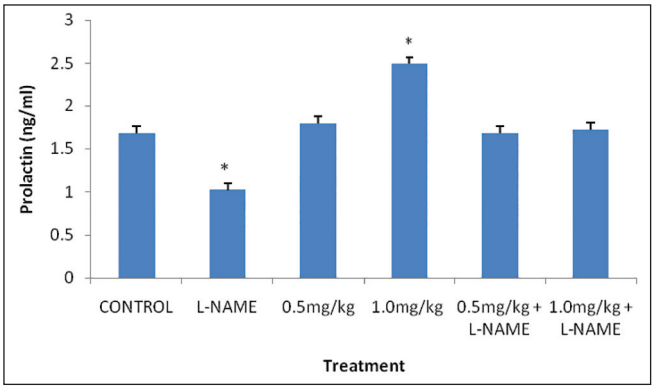

Figure 4: Serum prolactin level in male rats treated with nicotine and L-NAME. Values are expressed as mean ± SEM of 8 rats. * = $P < 0.05$ vs control

biological systems, including reproductive hormone regulation.[11,17,24] This study shows no significant change in BW with L-NAME treatment. This observation is consistent with previous study.[25] Previously, nicotine administration has been associated with distortion of the pituitary and gonadal axis resulting in low serum testosterone and FSH, increased LH and prolactin, as well as decreased reproductive ability.[3] This study presents data indicating that L-NAME prevented nicotine-induced testosterone suppression when coadministered with nicotine.

We suggest that the ameliorative effect of L-NAME on nicotine induced altered testosterone is primarily, although not completely, due to a direct gonadal effect on testosterone secretion largely at the Leydig cell level; however, the involvement of the pituitary cannot be totally ruled out. Our previous study has associated nicotine administration with increased serum and testicular NO; thus, L-NAME might have probably reduced the NO concentration which was harmful to the physiology of the testis. L-NAME alone did lead to an increase in testosterone, compared with control, although it did not quite achieve statistical significance. Thus, the notion that blockade of NOS by L-NAME causes a rise in testosterone (even in the absence of nicotine) is generally supported by our findings. This is in consonance with earlier studies.[12,26,27]

The rise in LH level observed in 1.0mg/kg BW was significantly reversed, thus implying that the increase in testosterone associated with coadministration of L-NAME and 10mg/kg BW led to negative feedback effect on the LH rise. The rise in LH in the L-NAME only treated group was observed in this study. This is in consonance with the study by Chatterjee *et al.*,[28] that L-NAME enhanced GnRH-induced LH release from pituitaries of rats but contrary to the studies of Ceccatelli.[29] There are also other conflicting reports indicating that NO facilitates[30] or has no effect[14] on LH release; hence, further studies are needed to elucidiate the cause of this differing data. However, there are reasons to believe that the NO system participates in the physiological control of the male mammalian reproductive system. For example, neurons expressing NOS mRNA have been found in the diagonal band of Broca, an area rich in LHRH neurons. The NOS mRNA expressing cells were seen to surround the LHRH cells.

This provides anatomical support for the idea that NO might regulate LHRH physiology in a paracrine manner at the level of the LHRH producing cell body.[31] In another study, NOS containing fibers were seen in the hypothalamic median eminence, sometimes adjacent to LHRH secretory neuron terminal. Such anatomical data suggest a paracrine role for NO in LHRH physiology at secretory terminals as well.[27] The serum level of prolactin in the L-NAME treated rats was significantly decreased and coadministration of L-NAME with nicotine reversed the observed hyperprolactinemia in nicotine-treated animals. This observation is in agreement with previous studies that observed decrease in basal prolactin level with L-NAME administration.[8,32,33] This might be attributed to the ability of L-NAME to increase the turnover and release of dopamine through occupying mu or kappa receptors of endorphins,[34,35] since reduction of dopamine could lead to overproduction of prolactin by lactotroph cells.[36]

Hyperprolactinemia impairs male gonadal functions by acting at various levels, possibly by decreasing gonadotropin releasing hormone (GnRH) pulse generator activity and/or by decreasing LH and FSH secretion;[37] hence, the reversal of the decreased FSH level observed when animals were treated with L-NAME and nicotine could be connected with the ability of nicotine to prevent hyperprolactinemia in nicotine-treated male rats.

Conclusion

In summary, our data show that the suppressive effects of nicotine on testosterone level of the adult male rat can be prevented by NOS blockade with L-NAME. It appears that these beneficial effects were mediated primarily within the gonad probably at the Leydig cell level. However, the involvement of the pituitary cannot be totally ruled out. Taken together, these findings seem to indicate that NOS blockade can act independently at a gonadal level. This study may support the concept that the use of NOS inhibitor in infertile male smokers may be useful.

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