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Title: Tadalafil Preserves Penile Nitric Oxide Synthase from Detrimental Effect of Paroxetine in Rats

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Objective: Paroxetine is a commonly prescribed SSRI that can impair erectile function in animal models via inhibition of nitric oxide synthase (NOS). Tadalafil potentiates nitric oxide (NO)-mediated responses in isolated trabecular smooth muscle and penile erection. The purpose of this study was to evaluate the impact of co-administering tadalafil with paroxetine on penile NOS levels in rats.

Materials and Methods: A total of 30 male Sprague-Dawley rats were divided into 3 groups as control (Group-C), paroxetine (Group-P) and paroxetine plus tadalafil (Group-P+T). After 28 days of treatment, rats were sacrificed and their penile tissues were harvested for analysis. NOS isoform protein levels and immunoreactivity scores of NOS were assessed. Statistical significance level was set at $p < 0.05$.

Results: Neuronal NOS (nNOS) levels were significantly decreased in group-P, compared with group-C ($P < 0.001$). In comparison, rats in group-P+T had significantly higher nNOS levels compared to group-P ($P < 0.001$). Endothelial NOS (eNOS) and inducible NOS (iNOS) levels were significantly higher in group-P compared with group-C ($P < 0.01$). The levels of eNOS and iNOS in group-P+T were similar to group-C.

Conclusion: Daily treatment with tadalafil prevented chronic paroxetine-induced changes in all three NOS isoform levels. Tadalafil treatment may therefore be a useful therapy in men with paroxetine-associated erectile dysfunction.

Keywords: Erectile dysfunction, Tadalafil, Paroxetine, Nitric oxide, Rat

Introduction

Antidepressants are frequently prescribed class of drugs. While major depressive disorder is the original intended target of antidepressant medications, they have been increasingly used off label for obsessive-compulsive disorder, generalized anxiety disorder, other psychiatric conditions and premature ejaculation [1]. Selective serotonin reuptake inhibitors (SSRIs) result in increased serotonin levels by preventing reuptake of this neurotransmitter. However, SSRIs are associated with side effects including erectile dysfunction (ED), which affects up to 50% of men using SSRIs [2]. Not all

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SSRIs result in ED at comparable frequencies. Namely, paroxetine is a SSRI that is associated with a high frequency of ED [3].

Previous studies have linked paroxetine's inhibition of nitric oxide synthase (NOS) with its propensity to cause ED [4, 5]. There are three types of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NOS catalyzes the synthesis of nitric oxide (NO) from L-arginine, and NO activates guanylate cyclase enzyme, resulting in increased cyclic guanosine monophosphate (cGMP) levels inside the endothelial smooth muscle cells. These events facilitate penile corporal smooth muscle relaxation, increase blood flow into the corpora cavernosa, and consequently results in a firm erection [6].

Phosphodiesterase type 5 (PDE5) inhibitors are commonly used for the treatment of ED. Like other phosphodiesterase inhibitors, PDE5 inhibitors prevent cGMP hydrolysis, leading to higher cGMP levels. Uniquely, PDE5 is localized to the corpus cavernosum and thus PDE5 inhibitors facilitate erection without significant systemic vasodilation [7]. Tadalafil is a highly effective PDE5 inhibitor, with even low daily doses improving erectile function in up to 86% of patients [8].

Paroxetine can decrease NOS levels and tadalafil works in the NO cascade to increase levels of downstream products. Therefore, concomitant administration of the two drugs might mitigate the paroxetine-dependent changes in NOS levels. Here we present data examining eNOS, iNOS and nNOS levels in penile tissue as a function of paroxetine and tadalafil treatment.

Materials and methods

Animals

After obtaining Institutional Animal Care and Use Committee (IACUC approval No. 2014/12), 30 7-week-old male Sprague-Dawley rats weighing 250-300 grams were randomly allocated into 3 groups of 10. Rats were allowed unlimited water, fed standard chow, and were kept at 22°C with 12 hours of light and 12 hours of dark per day. Daily for 4 weeks, the rats were administered medications orally between the hours of 12:00-14:00. In the control group (Group C), rats were administered a placebo of 5ml/kg tap water. Group P was given 20mg/kg paroxetine dissolved in 5ml/kg tap water. The paroxetine + tadalafil group (Group P+T) was given the paroxetine solution plus 5mg/kg tadalafil (5 mg tadalafil dissolved in 1 ml tap water).

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On the 29th day of the protocol, all rats were sacrificed. First, the rats were anesthetized using a combination of 5mg/kg xylazine, followed by 65mg/kg ketamine administered intramuscularly 15 minutes later. Each rat was shaved and placed in the supine position. A suprapubic vertical incision was used to access and excise the penis down to the level of the pubic symphysis. Subsequently, pentobarbital dosed at 150 mg/kg was administered. The distal 1/3 of the resected penile tissue was used for immunohistochemical analysis whereas the proximal 2/3 was sectioned in phosphate buffered saline (PBS) in a petri dish placed on ice (NaCl-phosphate; 0.2M, pH 7.29) for ELISA analysis.

Histopathological Analyses

The distal portions of the rat penis were fixed in 10% buffered formaldehyde solution for 1 day. Tissues were embedded in paraffin blocks and 4mm thick sections were made. Lysine coated slides were prepared and immunohistochemical analyses with nNOS (ab3511, polyclonal, Ig G isotype, UK), iNOS (ab204017, polyclonal, Ig G isotype, UK), and eNOS (ab50010, polyclonal, Ig G isotype, UK) antibodies were performed. All antibodies were diluted with a 1:100 ratio. Sections were examined for staining intensity by a pathologist (SA) who was blinded to the rats' group. Semi-quantitative scoring was used where 0=none, 1=mild, 2=moderate and 3=intense staining.

NOS Enzyme Levels

Cavernosal tissues were excised, and the urethral tissues were excluded. The cavernosal tissues were diced into small pieces in cold PBS and homogenized using a homogenizer (Ultra Turrax Type T25-B, IKA Labortechnik, Germany) at 16,000rpm for 3 minutes on ice in buffer. Afterwards, they were re-homogenized with a glass homogenizer on ice and were subjected to two freeze thaw cycles followed by ultrasonication. The resulting homogenates were centrifuged at 5,000rpm for 30 minutes at -20°C. Protein levels of NOS isoforms (nNOS, eNOS, and iNOS) were evaluated using commercial ELISA kits (Cloud-Clone Corp., Houston, TX) at 450 nm using a spectrophotometer (BioTek ELISA reader model, Winooski, VT) by using 100µl homogenate. Results were calculated in ng/ml.

Statistics

Immunohistochemical data and differences in NOS levels were examined using one way analysis of variance (ANOVA) and post-hoc Tukey test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA). Results were expressed as mean \pm SD, with $p < 0.05$ considered significant.

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Results

Histopathological Analyses of NOS Levels in Corpus Cavernosum

Immunohistochemical analyses of corporal cavernosal tissues are shown in Figure 1. Semi-quantitative staining intensity scoring of cavernosal tissues revealed that nNOS levels were significantly lower in Group P when compared to Group C (0.6 ± 0.69 vs. 2.2 ± 0.42 , $p < 0.001$) whereas co-administering tadalafil with paroxetine was associated with preserved nNOS levels in Group P+T (1.7 ± 0.48) which was similar to Group C ($p = 0.122$). Corporal eNOS levels were significantly higher for Group P compared to Group C (2.8 ± 0.42 vs. 2.1 ± 0.31 , $p = 0.003$), but there was no significant difference in eNOS levels between Group P+T (2.4 ± 0.51) and each of Group C ($p = 0.274$) and Group P ($p = 0.109$). Moreover, iNOS levels in Group P were significantly higher compared to group C (2.6 ± 0.51 vs. 1.9 ± 0.56 , $p = 0.012$) whereas they were similar in Group C vs. Group P+T (1.9 ± 0.56 vs. 2.2 ± 0.42 , $p = 0.393$) and Group P vs. Group P+T (2.6 ± 0.51 vs. 2.2 ± 0.42 , $p = 0.199$).

ELISA Analyses NOS Levels in Penile Tissues

NOS isoform protein levels in penile tissues were determined using ELISA (Table 1, Figure 2). Group P had significantly lower nNOS than group C ($p < 0.001$). Simultaneously administering paroxetine with tadalafil was able to preserve the nNOS levels in Group P+T, which were significantly higher than those in Group P ($p < 0.001$). Group P also had significantly higher eNOS ($p < 0.01$) and iNOS ($p < 0.01$) levels than Group C. No difference in eNOS ($p = 0.122$) and iNOS ($p = 0.226$) levels was observed in Group P+T compared to the Group C.

Discussion

In the United States, there is a growing population treated each year for mental illnesses [9]. As a result, there is a concomitant rise in the prescription and use of psychotropic medications, in particular antidepressants [10-12]. In 2010, there were 253.6 million antidepressant prescriptions totaling approximately \$11 billion dollars in medication costs [13, 14]. However, the use of antidepressants is not without side effects. In men, antidepressants can impair fertility and semen quality [15-18]. Moreover, numerous antidepressants (particularly SSRIs), can also dramatically

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impact libido and sexual function. Several animal studies have shown their negative effects on erectile function [5, 19-21].

The mechanism by which these drugs affect sexual function is incompletely understood. Both dopamine and serotonin are believed to be involved in sexual function, with dopamine having a protective effect and serotonin having an inhibitory effect [22]. However, serotonin levels alone cannot account for the sexual side effects of SSRIs, as all SSRIs increase serotonin levels similarly, but some—such as paroxetine—have a much higher prevalence of sexual side effects. Moreover, increases in NADPH oxidase or reactive oxygen species resulting in a decrease in NO bioavailability may also be responsible for SSRI-induced ED [23]. However, paroxetine seems to have a unique ability to inhibit NOS [5].

NOS, specifically nNOS, is a recent area of interest in erectile function studies [4, 24]. Contrary to predictions, rats deficient in nNOS had normal sexual and erectile function, which may be due to compensatory, protective overexpression of eNOS [4]. Angulo et al. [5] used cavernosal nerve electrical stimulation to create frequency-dependent intracavernosal pressure (ICP) increases. They found that NOS inhibitors, acute and chronic paroxetine treatment all caused decreased ICP responses. In contrast, citalopram-treated rats responded similarly to the control group [5]. This is believed to be due to the structural similarity of paroxetine, but not citalopram, to the cytochrome P450 isoenzymes, which allows paroxetine to inhibit NOS [25, 26]. Similarly, chronic treatment with paroxetine, but not citalopram, caused a significant decrease in erectile response. Additionally, in the paroxetine treated rats plasma nitrite and nitrate levels were decreased by 61% ($p < 0.05$), while the citalopram treated rats had normal plasma nitrite and nitrate levels when compared to the controls. When examining protein levels in penile tissues, nNOS levels were decreased by 31% in the paroxetine treatment group. nNOS levels were not affected by citalopram treatment, and eNOS levels were not significantly changed in either group. Thus, both acute and chronic paroxetine treatment may lead to an inhibition of nNOS and therefore NO production causing a reduction in plasma NO derivatives and a decreased ICP response to cavernous nerve stimulation [5]. It is important to note that Angulo et al. [5] did not find a compensatory eNOS increase associated with decreased nNOS levels, as observed in other studies [4]. A possible explanation for this difference is that the rats lacked nNOS only after they were developmentally mature and therefore could not upregulate eNOS expression like nNOS knock out rats, which lacked the enzyme from conception [5]. On the other hand, the compensatory rise in eNOS could suggest an inhibitory function.

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In our study, nNOS levels decreased in the corpus cavernosum with paroxetine treatment but combined treatment with tadalafil preserved the nNOS levels in both ELISA homogenates and immunohistochemical staining. Moreover, our analyses demonstrated an increase in eNOS and iNOS levels with paroxetine treatment, which is prevented by co-treatment with tadalafil. In a similar study, Kadioglu et al. [20] studied electrical field stimulation (EFS) induced relaxation of the penile tissues, which is mediated by NO. They found that both paroxetine and the NOS inhibitor L-NAME inhibited relaxation of penile tissue. The group hypothesized that paroxetine decreases NO production by acting as an inhibitor of either eNOS or nNOS. On the other hand, sertraline and fluoxetine were found to increase EFS induced relaxation of penile tissue via a relaxing factor—possibly NO [20]. The detrimental effect of paroxetine on erectile function has also been demonstrated in another study of Angulo et al. [27] who found that acute treatment with paroxetine significantly reduced ICP increase in response to cavernous nerve stimulation when compared with the control group. Conversely, vardenafil significantly potentiated the stimulation dependent ICP increase compared to the control group. The authors recorded that administering paroxetine and a PDE5 inhibitor (vardenafil) together led to a similar ICP response as that seen in the control group [27].

This study is not without limitations. Comparing the ICP measurement outcomes among the three groups could support the therapeutic effect of daily tadalafil treatment in paroxetine induced penile changes. Similarly, analyses of the phosphorylation status of NOS isoforms might reveal other mechanism associated with the impact of paroxetine and tadalafil on cavernosal tissues. Moreover, assessment of NOS isoforms in penile endothelial cell, smooth muscle cells and neuronal cells separately by immunohistochemistry methods would provide a better insight about the extend of paroxetine induced NOS alterations. Therefore, future molecular and further studies must be carried out in order to elucidate the mechanisms by which paroxetine and tadalafil affect NOS expression levels.

In conclusion, the increase in general psychological disorders and common use of SSRIs, namely paroxetine, is associated with sexual side effects, which include ED. Our data suggest that daily tadalafil may be a useful adjunct therapy, in concert with paroxetine, to prophylactically prevent unwanted sexual side effects and increase patient satisfaction with their treatment regimen in men with depression and premature ejaculation or other illnesses such as paraphilias, chronic pain syndromes.

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Table 1: The nNOS, eNOS and iNOS levels of groups in ELISA

nNOS			eNOS			iNOS		
Group-C	Group-P	Group-P+T	Group-C	Group-P	Group-P+T	Group-C	Group-P	Group-P+T

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Mean	3.578	1.963	3.508	3.154	3.760	3.410	2.894	3.448	3.190
± SD	0.276	0.187	0.186	0.220	0.230	0.423	0.286	0.263	0.321

Figure-1: Immunohistochemical studies in the cavernous tissue. Enhanced eNOS staining (red arrow) in cavernous sinus endothelial cells can be seen in Group P (B, x200). Moderate eNOS immunoreactivity is observed in the endothelial cells in Group P+T (C, x200). Similarly, strong iNOS staining (yellow arrows) is observed in Group P (E, x200) whereas less immunoreactivity was observed in Group P+T (Fx200). Finally, nNOS immunoreactivity was the greatest in the endothelial cells of the Group C (G, x400) (green arrows). Decrease in nNOS staining in the endothelial cells of Group P (H, x400) and enhanced nNOS staining in Group P+T can be observed (Ix400).

Figure-2: NOS isoform levels measured by ELISA.

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