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Sildenafil Corrects the Increased Contractility of Rat Detrusor Muscle Induced by Alprostadil In-Vitro

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ABSTRACT

Background: Sildenafil (PDE5-inhibitor) and alprostadil (PGE₁) are used in combination clinically for the management of some cases of erectile dysfunction. Despite the roles of prostaglandins (PG) and NO pathways in contractility of bladder smooth muscle are frequently studied, the effect of sildenafil/alprostadil combination and the crosstalk between NO/cGMP and PG pathways on bladder activity is not documented.

Methods: Organ-bath experiments were performed using isolated rat detrusor muscle. Direct and neurogenic contractions were induced using ACh and electric stimulation (EFS, 4Hz, 80V, 1ms), respectively. The contractile responses in absence and presence of the tested drugs at different concentrations were compared. Results are expressed as mean $\pm$ SEM (n=5-7).

Results: Alprostadil (0.01-10 μ M) concentration-dependently potentiated ACh (100 μ M)- and EFS (4Hz)- induced contraction. Maximum potentiation of ACh-contraction in presence of alprostadil was 40 $\pm$ 5%. Sildenafil potentiated ACh-induced contraction at low concentrations (0.01-1 μ M), but inhibited it at higher ones (10-100 μ M). IBMX (non-selective PDE-inhibitor, 10nM-100 μ M) and SNP (NO-donor, 1nM-1mM) produced the same biphasic pattern. The potentiatory phase of sildenafil was inhibited by atropine (0.1 μ M), L-NAME (non-selective NOS-inhibitor, 100 μ M), N-PLA (nNOS-inhibitor, 30 μ M) or MB (nonselective GC-inhibitor, 10 μ M). In presence of sildenafil (0.1 μ M), the concentration-response curve of alprostadil (0.01-10 μ M) on both ACh and EFS-induced contraction was clearly shifted downward.

Conclusions: A crosstalk between PGE₁ and NO/cGMP pathways may exist. At low concentrations only, the effect of sildenafil on bladder contractility is dependent on NO/cGMP. cGMP intracellularly-elevated by sildenafil, may inhibit the activity of PLC and hence the cascade of EP₁-receptors, thus masking the hyperactivity of bladder caused by alprostadil, which adds to the advantages of this combination.

Keywords: sildenafil; alprostadil; PGE; urinary bladder; detrusor muscle; NO/cGMP pathway

1. Introduction

The urinary bladder is the organ responsible for storage of urine and micturition. The bladder is a tetrahedron organ, when empty it lies extraperitoneal behind the pubic area [1]. The bladder is divided into two main parts; (a) the body which is located above the orifice of the ureter and (b) the base which consists of the trigone and the bladder neck [2]. The serosa is the outer layer, the detrusor smooth muscle constitutes the body of the bladder, and the urothelium is the innermost layer lining the bladder wall [3]. The major neurotransmitters involved in contractility of detrusor smooth muscles are acetylcholine (ACh) and adenosine triphosphate (ATP). Muscarinic and purinergic receptors activation cause elevation of intracellular calcium (Ca^{2+}) level and activation of voltage-dependent L-type Ca^{2+} channels resulting in contraction of the muscle [4]. The contractile receptors in the detrusor muscles include; muscarinic receptors (M_2 and M_3) which are the dominant ones, and purinergic receptors (P2X_1). M_3 receptors are the main contractile component. However, M_2 receptors are the more expressed ones and are responsible mainly for the recontraction of the detrusor muscles after relaxation [5, 6].

Bladder overactivity and hypoactivity are among the most common disorders of the lower genitourinary tract, in addition to inflammatory conditions and cancer [7]. Overactive bladder (OAB) is defined by the International Continence Society as urinary urgency with accompanying increased daytime urinary frequency and nocturia with or without urgency urinary incontinence [8]. The exact role of endogenous prostaglandins (PGs) in tissues of lower urinary tract is not well elucidated, but various studies have confirmed that exogenous PGs alter the bladder motor activity and micturition reflex in both human and animals [9-12]. The main PGs synthesized in bladder under physiological and pathological conditions are PGE_2 , $\text{PGF}_{2\alpha}$, PGI_2 and TXA_2 [13]. EP_1 and EP_3 receptors are reported to be involved in bladder contraction, while EP_2 and EP_4 induce relaxation [14]. PGE_2 level was found to be higher in patients with overactive bladder (~~OAB~~) [15], an effect mainly mediated via EP_1 receptors. Alprostadil is a synthetic analog of prostaglandin E_1 (PGE_1). Its main uses involve the management of erectile dysfunction [16] and pulmonary hypertension [17]. Alprostadil has been first demonstrated by Ishii et al. for treatment of erectile dysfunction [18]. However, it has not been approved by the Food and Drug Administration until 1995 when it has been marketed under the brand name Caverject® as intracavernous injection. A year later, it was approved for usage by intraurethral route under the brand name Muse® [16] Alprostadil is also used with success in the management of patent Ductus Arteriosus, intermittent

claudication, Buerger's disease and Raynaud's phenomenon [19-22]. PGE₁ is reported to provoke strong micturition reflexes in dogs and in rabbits [23, 24], and induces contraction of human detrusor muscles [25]. Increased urinary urgency in humans is in fact reported as a side effect of alprostadil [26, 27].

The exact role of nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway, on the other hand, is not yet fully understood. Previous studies have reported that NO induces urinary bladder relaxation via guanylyl cyclase (GC)/cGMP pathway [28], while other studies reported that NO enhances detrusor muscle spontaneous contractility via cGMP-independent mechanism [29]. Sildenafil (Viagra[®]) - used to manage erectile dysfunction and approved by FDA for the management of pulmonary hypertension- is one of the major modulators of NO. Sildenafil is a phosphodiesterase 5 (PDE-5) inhibitor; it inhibits the breakdown of cGMP thus prolongs the action of NO in smooth muscles [30]. Gumrah et al. (2017) proved that sildenafil reduced bladder contractility in rats and Aizawa et al. (2017) showed that sildenafil may improve bladder storage in female rats by exhibiting an inhibitory role on the bladder afferent transduction [31, 32].

Lower urinary tract symptoms including erectile dysfunction are frequent problems in older men worldwide, they were reported to be close to 95% of the population aged over 60 in a survey of 30,000 patients in the USA, UK and Sweden [33]. In 2008, almost 45.2% of the worldwide populations have been reported to be affected by at least one lower urinary tract symptom including 10.7% suffering from OAB. The prevalence of lower urinary tract symptoms is expected to be higher in Middle East and estimated to increase by almost 18.4% in 2018 [34]. Both alprostadil and sildenafil are considered the mainstay of management of erectile dysfunction, and are clinically used in combination in cases unresponsive to sildenafil alone [35, 36]. The effect of sildenafil/alprostadil combination and the potential crosstalk between NO/cGMP and PG pathways on detrusor muscle contractility as an important component of bladder function is not previously investigated. Therefore, this study aims to evaluate the effect of combining alprostadil and sildenafil on rat detrusor muscle contractility and to investigate the potential mechanism(s) controlling their interaction.

2. Material and Methods:

2.1. Materials

The chemicals used in this study and their respective sources were as following: (3)-isobutyl-1-methyl xanthine (IBMX, Sigma), Acetylcholine chloride (ACh, Sigma), alprostadil

(Tocris), Atropine sulphate (Merck), Methylene blue (MB, Sigma), NG-Nitro-L-arginine methyl ester (L-NAME, Sigma), N ω -propyl-L-arginine hydrochloride (N-PLA, Tocris), Sildenafil citrate (Viagra®, Pfizer), Sodium nitroprusside (SNP, Merck), Thiopentone sodium (vial, Biochimie).

Stock solutions (2.22 mg/ml) of IBMX and (1.4 mg/ml) alprostadil were prepared in 95% ethanol and kept frozen. The obtained final bath concentration of ethanol was less than 1% v/v which showed no significant effect on the studied tissue. Stock solutions (6.76 mg/100 ml) of atropine sulphate, (0.76 mg/ml) of N-PLA, (26.2 mg/ml) of SNP and (6.67 mg/ml) of sildenafil citrate were prepared in distilled water and kept frozen. SNP was kept in dark bottle and subsequent dilutions were made in aluminum foil-covered vials. Solutions (0.32 mg/ml) of MB and (2.7 mg/ml) of L-NAME were prepared in distilled water and kept frozen. A fresh solution (18 mg/ml) of ACh (Sigma) was prepared daily in Krebs solution.

2.2. The isolated rat detrusor smooth muscle

Experiments were carried out on male albino Wistar rats weighing 250–270 g. The animals were obtained from the [Alexandria University](#) Faculty of [Pharmacy](#) Animal House. The rats were housed in animal cages, in groups under normal environmental conditions of day light and temperature with access to water and chow. Study protocols comply with the guidelines for the proper conduct of animal experiments and are approved by the local Animal Care and Use Committee (ACUC). All applicable international, national, and institutional guidelines for the care and use of animals were followed. Preparation of the muscle was performed as described by Luheshi and Zar ~~(1991)~~ [37]. Rats were anesthetized by intraperitoneal injection of thiopentone (50 mg/kg) and then sacrificed by exsanguination. In order to expose and isolate the urinary bladder, the lower abdomen was opened and the bladder separated from associated connective tissues and blood vessels. The bladder was then excised above its trigone to isolate only the detrusor muscles of the bladder away from its neck and urethral sphincter, fixed in a petri dish and two lateral incisions along its longitudinal axis were made and the bladder was unfolded giving rectangular sheet which was cut longitudinally to give two strips of detrusor muscle 2x15 mm. The detrusor muscle was fixed at one end between two parallel platinum electrodes 4 to 5 mm apart and mounted in a 10 ml organ bath containing Krebs solution (Composition in mM; NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄.7H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11), kept at 37°C and continuously aerated with 95% O₂ and 5% CO₂. The other end was tied and attached to a

force displacement transducer (Grass FT-03) which in turn was connected to a computerized data acquisition system through an MLAC11 Grass adapter cable. Tension studies were performed using Lab Chart-7 pro software (Power Lab 4/35, model ML 866/P; AD Instrument Pty Ltd, Castle Hill, Australia). The tissue was left to equilibrate for 60 min under a resting tension of 0.5 g during which the physiological solution was replaced each 15 min. Meanwhile, time-matched control experiment was conducted over the duration of the experiment.

The contractile responses to ACh (in g tension) in absence and presence of the tested drug were measured and the percentage change from control was calculated. The half-maximum effective concentration (EC_{50}) value is calculated from the concentration response curve of the agonist. Changes in relative potency of ACh on detrusor muscles are determined by comparing EC_{50} values of ACh in absence and presence of the tested drug. For electric field stimulation (EFS) experiments, each strip was subjected to increasing frequencies (1-16 Hz; voltage 80 V, pulse duration 1 ms, 3 min interval) and submaximal frequency (4 Hz) was used for further experiments. Stimulation was provided using two parallel platinum electrodes which were attached to a Grass electronic stimulator (Model S 48). After incubation with the studied drug, the frequency response curve or the submaximal frequency was repeated. The contractile responses in absence and presence of the tested drug were measured and the percentage change from control was calculated.

First, modulators of NO/cGMP pathway were tested, where concentration response curves of sildenafil (10^{-8} - 10^{-4} M), IBMX (nonselective PDE inhibitor, 10^{-8} - 10^{-4} M) and SNP (10^{-9} - 10^{-3} M) were conducted and their effects on ACh (10^{-4} M) and EFS (4 Hz)-induced contraction were recorded and compared to time-matched control over the experiment duration. Effect of some selected blockers on sildenafil's action was investigated to determine its mechanism of action. This was followed by the evaluation of alprostadil/sildenafil combination in modulating rat detrusor muscles contractility.

2.3. Data Analysis and Statistics

All the obtained results are expressed as mean $\pm$ SEM. Values between parentheses (n) represent the number of animals in each group. For analysis of unpaired data, ~~the student's t-test~~ nonparametric Mann-Whitney U test was used, while for multiple comparison, one-way analysis of variance (ANOVA) was used followed by Kruskal-Wallis test ~~Dunnett's post-test~~.

Statistical significance was considered at the level of $p < 0.05$. Analysis was performed using computer software program GraphPad Prism 6.01.

The contractile responses to ACh or EFS (in g tension) in absence and presence of the tested drug were measured and the percentage change from control was calculated as follows;

$$\% \text{ Change in contraction} = \frac{\text{Contraction (in presence of tested drug)} - \text{Contraction (in absence of tested drug)}}{\text{Contraction (in absence of tested drug)}} \times 100$$

3. Results:

3.1. Effect of sildenafil, IBMX and SNP on ACh and EFS-induced contraction

Sildenafil caused a significant potentiation of ACh-induced contraction at lower concentration with a percentage potentiation of $10.47 \pm 2.60\%$ at 10^{-8} M sildenafil, while upon increasing sildenafil concentration to (10^{-5} and 10^{-4} M), it started to cause a significant inhibition of ACh-induced contraction. Sildenafil (10^{-4} M) significantly inhibited ACh-induced contraction by $76.96 \pm 2.00\%$. In case of EFS, sildenafil caused a significant potentiation of EFS-induced contraction at lower concentrations (10^{-7} - 10^{-6} M) with a maximum attained potentiation of $31.53 \pm 7.62\%$ at 10^{-6} M, and significantly inhibited EFS-induced contraction at high concentration (10^{-4} M) by $44.40 \pm 2.72\%$ ($p < 0.05$) (Figure 1). The same biphasic pattern appeared using IBMX which caused a significant potentiation of ACh-induced contraction at low concentrations (10^{-7} and 10^{-6} M) with a maximum attained potentiation of $25.87 \pm 5.02\%$ at 10^{-6} M, while upon increasing IBMX concentration to (10^{-5} and 10^{-4} M), it started to cause an inhibition; IBMX (10^{-4} M) significantly inhibited ACh-induced contraction by $42.47 \pm 6.10\%$. In case of EFS, IBMX caused a significant potentiation of EFS-induced contraction at low concentrations (10^{-8} - 10^{-6} M) with a maximum attained potentiation of $41.44 \pm 3.31\%$ at 10^{-6} M, followed by a significant inhibition at higher concentrations (10^{-5} and 10^{-4} M). IBMX (10^{-4} M) significantly inhibited EFS-induced contraction by $44.64 \pm 4.82\%$ ($p < 0.05$) (Figures 2 & 3).

Similarly, SNP caused a significant potentiation of ACh-induced contraction at low concentrations (10^{-9} - 10^{-6} M) with a maximum attained potentiation of $26.05 \pm 4.18\%$ at 10^{-6} M. Upon increasing SNP concentration to 10^{-3} M, it significantly inhibited ACh-induced contraction reaching a maximum of $13.27 \pm 1.89\%$. In case of EFS, SNP also caused a significant potentiation of EFS-induced contraction at low concentrations (10^{-9} - 10^{-6} M) with a maximum attained potentiation of $22.63 \pm 2.19\%$ at 10^{-6} M, followed by a significant inhibition

at higher concentration where SNP 10^{-3} M caused $23.43 \pm 3.26\%$ inhibition ($p < 0.05$) (Figures 4 & 5).

3.2. Modulatory role of atropine, L-NAME, N-PLA and methylene blue on sildenafil's action on EFS-induced contraction

Effect of atropine (0.1 μ M), L-NAME (NO synthase 'NOS' inhibitor, 10^{-4} M) [38], N-PLA (neuronal NOS 'nNOS' inhibitor, 3×10^{-5} M) [39] or MB (nonselective GC inhibitor, 10^{-5} M) [40] on sildenafil's action on EFS-induced contraction of rat detrusor muscles was tested. Atropine significantly inhibited EFS-induced contraction by $30.19 \pm 5.92\%$, while both L-NAME and MB significantly potentiated EFS-induced contraction by $16.38 \pm 3.18\%$ and $20.23 \pm 2.82\%$ respectively. On the other hand, N-PLA produced no significant effect ($p < 0.05$). In combination with sildenafil, all the tested blockers or inhibitors (atropine, L-NAME, N-PLA and MB) caused a significant inhibition of sildenafil-induced potentiation at concentrations (10^{-8} - 10^{-5} M) of sildenafil, i.e. the potentiatory effect of sildenafil was masked. As for the higher concentration (10^{-4} M) of sildenafil which inhibited EFS-induced contraction; atropine produced no significant effect, while L-NAME, N-PLA and MB significantly potentiated the inhibitory effect of sildenafil ($p < 0.05$) (Figure 6).

3.3. Effect of sildenafil on alprostadil-induced potentiation of ACh and EFS-induced contraction

Effect of alprostadil (10^{-9} - 10^{-5} M) was tested on the contractile effect of a submaximal ACh concentration of 10^{-4} M and a submaximal frequency of 4 Hz on rat detrusor muscles and compared to time-matched control over the duration of the experiment. Alprostadil caused a concentration-dependent potentiation of both ACh and EFS-induced contraction with a maximum attained potentiation of $40.01 \pm 5.29\%$ and $25.32 \pm 4.61\%$ respectively in presence of 10^{-5} M alprostadil. A concentration of 10^{-7} M sildenafil which produced a submaximal potentiatory effect of $28.21 \pm 3.9\%$ on EFS-induced contraction was chosen and its effect on alprostadil-induced potentiation of ACh and EFS-induced contraction of rat detrusor muscles was tested.

Sildenafil caused a significant inhibition of alprostadil's potentiatory effect of both ACh and EFS-induced contraction at concentrations (10^{-8} - 10^{-5} M) of alprostadil. Sildenafil significantly decreased the maximum attained potentiation of ACh-contraction induced by 10^{-5} M alprostadil from $40.01 \pm 5.29\%$ to $23.53 \pm 4.66\%$, but was still significant from control. On

the other hand, in case of EFS, sildenafil significantly decreased the maximum attained potentiation of EFS-contraction induced by 10^{-5} M alprostadil from $25.32 \pm 4.61\%$ to $6.84 \pm 5.77\%$ which became not significant from control i.e. sildenafil completely abolished the potentiatory effect of alprostadil even at the highest concentration ($p < 0.05$) (Figure 7).

4. Discussion

~~The bladder is divided into two main parts; (a) the body which is located above the orifice of the ureter and (b) the base which consists of the trigone and the bladder neck [32]. The serosa is the outer layer, the detrusor smooth muscle constitutes the body of the bladder, and the urothelium is the innermost layer lining the bladder wall [33]. The major neurotransmitters involved in contractility of detrusor smooth muscles are acetylcholine (ACh) and adenosine triphosphate (ATP). Muscarinic and purinergic receptors activation cause elevation of intracellular calcium (Ca^{2+}) level and activation of voltage-dependent L-type Ca^{2+} channels resulting in contraction of the muscle [34]. The contractile receptors in the detrusor muscles include; muscarinic receptors (M_2 and M_3) which are the dominant ones, and purinergic receptors (P_2X_1). M_3 receptors are the main contractile component. However, M_2 receptors are the more expressed ones and are responsible mainly for the recontraction of the detrusor muscles after relaxation [35, 36].~~

Prostaglandins have been reported to play a major role in lower urinary tract function. The exact role of endogenous PGs in different tissues of lower urinary tract is not well elucidated, but various studies have confirmed that exogenous PGs alter the bladder motor activity and micturition reflex in both human and animals [10]. It was originally shown that vesical distension and pelvic nerve stimulation and M_5 receptor activation, evoke a release of PGE_2 into the pelvic venous blood of anesthetized dogs [41-43]. In line, intra-arterial administration of PGE_2 induced a significant decrease of the threshold micturition volume [44]. Moreover, Kondo et al. (1983) reported that micturition was most frequently provoked in dogs by the intravenous administration a derivative of PGE_1 among various other prostanoids tested [23]. In the current study, alprostadil caused potentiation of both ACh and EFS-induced contraction, indicating that PGE_1 may be involved in cholinergic and neuronally-mediated contraction of detrusor muscles. This effect also explains the increased micturition reflexes observed in animals [23, 24], and increased urinary urgency reported in humans caused by alprostadil [26, 27]. These results are consistent with those previously reported by Palea et al. (1998) who showed that PGE_1 induces contraction of human detrusor

muscles [25]. Boie et al. ~~(1997)~~ have reported that PGE₁ effect involves the activation of all PGs receptors of the E-series (EP₄>EP₃>EP₂>EP₁) [45]. Taking into consideration that alprostadil potentiated the effect of ACh more than that of EFS, and that ACh –via activating M₅ receptors- induces release of PGE₂ in the urothelium [43], an additive activation of E-receptors can be assumed in the presence of alprostadil. M₃ receptors on detrusor muscle are G_q-coupled which activates phospholipase C (PLC) with the formation of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), while M₂ receptors are G_i-coupled which inhibits cyclic adenosine monophosphate (cAMP)/cAMP-dependent protein kinase (PKA) and both in turn result in elevating the intracellular Ca²⁺ level [5, 6]. Alprostadil in detrusor muscles may involve the activation of EP₁ and EP₃ receptors coupled to G_q pathway, with subsequent induction of IP₃ response [14]. EP₃ receptors may also act via activating G_i resulting in elevation of intracellular Ca²⁺ level [46].

Nitric oxide- on the other hand- is reported to induce relaxation of bladder neck and external urethral sphincter [47, 48]. Previous clinical studies showed that PDE-5 inhibitors, especially tadalafil, are useful for management of lower urinary tract symptoms, tadalafil has been approved in 2011 for management of benign prostatic hyperplasia [49] Besides, several studies reported the efficacy of tadalafil use in management of OAB symptoms and improving the quality of life in adults [50]. Reges et al. ~~(2013)~~ reported that the systemic reduction of NO (by i.v. injection of L-NAME) causes detrusor overactivity [51], which is consistent with the results of the current in-vitro study. In an attempt to clarify the possible role of NO in mediating the effect of PGs on rat detrusor muscles, the effect of NO modulators; sildenafil, IBMX (nonselective PDE inhibitor) and SNP (NO donor) on both cholinergic and neuronally-mediated contraction of detrusor muscles was investigated. All of the tested compounds showed the same pattern of effect on both ACh and EFS-induced contraction; the low concentrations of the tested compounds caused a slight albeit significant potentiation of both ACh and EFS-induced contraction, while at high concentrations, they showed a strong significant inhibition. Although statistically significant, the inhibitory effect of SNP is much lower than that of sildenafil and IBMX. This finding supports the previous reports by Moon ~~(2002)~~ and Yanai et al. ~~(2008)~~ who suggested that NO may cause relaxation of human and guinea pig detrusor muscles preceded by contraction and this contractile effect is cGMP-independent involving Ca²⁺ release from intracellular stores [29, 52]. Moreover, Longhurst et al. ~~(1997)~~ showed that selective PDE-5 inhibitor potentiated cholinergic-mediated contraction of guinea pig detrusor muscles [53]. In contrast, other studies have reported that NO provokes

only a relaxant effect on human detrusor muscles [28]. A report by Oger et al. (2010) showed that sildenafil induces human detrusor muscle relaxation via cGMP and cAMP-dependent pathways and also involves cross talking with potassium (K^+) channels signaling pathway [54], and Aizawa et al. (2017) showed that the NO/cGMP pathway plays an inhibitory role on the bladder afferent nerve transduction [32].

In the current study, the effect of sildenafil in presence of L-NAME (nonselective NOS inhibitor) or N-PLA (selective nNOS inhibitor) was investigated. In presence of each inhibitor, the potentiatory effect of low concentrations of sildenafil was inhibited, suggesting that it may be actually NO-dependent, while the inhibitory effect of the high concentrations of sildenafil was unexpectedly potentiated rather than inhibited, indicating the involvement of mediators other than NO in the effect of these higher concentrations. Moreover, MB (nonselective guanylate cyclase GC inhibitor) showed the same pattern of effect as L-NAME and N-PLA; it inhibited sildenafil's potentiatory effect and potentiated its inhibitory effect on rat detrusor muscles, indicating that the potentiatory effect of sildenafil on detrusor contractility may be not only dependent on NO but also on cGMP levels which may activate voltage-independent Ca^{2+} channels and inward current upon activation of muscarinic receptors [55]. In order to investigate the involvement of muscarinic receptors in sildenafil's action, the muscarinic receptors were blocked using atropine in a concentration that causes less than 50% blockade. It was observed that, in presence of atropine, the potentiatory effect of sildenafil on rat detrusor muscles was inhibited, while its inhibitory effect was not affected. These results suggest that the potentiatory effect of sildenafil- which is NO/cGMP dependent- may depend on stimulation of inward Ca^{2+} current via muscarinic receptors activation. We cannot exclude that muscarinic receptor activation, specifically M_5 , is a driver for NO release from the urothelium [56], which may explain this observation concerning atropine blocking the NO-dependent phase of sildenafil. This observation also proves that sildenafil at higher concentrations acts by a mechanism that is not only NO/cGMP-independent but also cholinergically-independent. Since sildenafil is known to possess other mechanisms of actions such as inhibition of adrenergic alpha receptors in tissues of the lower genitourinary system [57], we can assume that in absence of NO and/or cGMP, the effect of sildenafil on alpha receptors in bladder cells becomes predominant leading to the observed increased relaxation. It is to be mentioned that alpha-1 receptors are reported to be expressed in the urothelium of pigs and that they mediate increases in the contractile rate and tension of the bladder [58], however; this assumption remains to be further investigated.

The results of the current study showed that the potentiatory effect of alprostadil on ACh and EFS-induced contraction was inhibited in presence of sildenafil (low concentration) by almost 40% and 73% respectively. This indicates that there may be a crosstalk between PGE₁ and NO/cGMP pathway in detrusor muscles. At the cellular level, in detrusor muscles, EP₁ and EP₃ receptors may be the major contributors to the contractile effect of PGE₁-analogue. The EP₁ receptors are G_q-coupled which activates PLC resulting in formation of IP₃ and DAG which in turn increase the intracellular Ca²⁺ level resulting in increasing the contractile response [59]. On the other hand, EP₃ receptors may act via G_q and also G_i which inhibits cAMP and PKA activation resulting in decreasing detrusor muscles relaxation [14]. Therefore, sildenafil, upon activation of NO/cGMP pathway, results in elevating the intracellular level of cGMP and activating cGMP-dependent protein kinase (PKG) which interacts with G_q pathway and cause inhibition of PLC contracting activity [60], which may inhibit the molecular pathways of EP₁ and EP₃ receptors. On the other hand, **we cannot exclude the possible action of alprostadil on EP₂ receptors which are G_s-coupled leading to the activation of adenylyl cyclase (AC)/cAMP pathway and hence relaxation, this latter could have been masked in detrusor muscle by the stronger effect on EP₁ and EP₃ leading finally to contraction.** Moreover, PKG is reported to activate PKA in smooth muscles [60] **thus potentiating the probable relaxatory action of alprostadil on EP₂, in addition to the inhibition of the molecular pathway of the contracting EP_{1/3} receptors (Figure 8).** All these events may eventually result in inhibition of the potentiatory effect of alprostadil on detrusor muscles by sildenafil. Interestingly, Dveksler et al. (1989) reported that EFS of the rat urinary bladder provoked greater release of tissue PGE₂ compared to basal levels [61], which may explain why the effect of the combination was more pronounced in experiments involving neurogenic stimulation. Measuring the level of cyclic nucleotides and NO as well as the expression of PGs receptors in detrusor muscles remains a limitation of the current study and requires further experiments to confirm the downstream pathway involved in the interaction of alprostadil with sildenafil in rat detrusor muscles.

Conclusions: Alprostadil, the synthetic PGE₁ potentiated neurogenic and muscarinic responses of rat detrusor muscle as an indication of potential increase in micturition responses, consistent with previous clinical reports. The potentiatory effect of alprostadil was significantly inhibited in presence of the PDE-5 inhibitor - sildenafil- indicating that PGE₁ pathway may have a crosstalk with NO/cGMP pathway. Elevated level of cGMP by sildenafil inhibits the activity of PLC involved in the pathway of alprostadil in detrusor muscles

reducing thus its stimulatory effect on detrusor muscle. This observation, if proved in humans, may greatly add to the advantages of the combinatory use of sildenafil and alprostadil in clinical practice for a better management of erectile dysfunction as well as pulmonary hypertension, for which many combinatory treatments are under research including the same combination.

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6. Conflict of Interest

Authors declare no conflict of interest

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8. Legends to figure

Figure (1). Representative tracings showing the effect of sildenafil (10^{-8} - 10^{-4} M) on **ACh** (10^{-4} M, panel a) or electric field stimulation (**EFS**, 4 Hz, panel b)-induced contraction of isolated rat detrusor muscles.

Figure (2). Effect of sildenafil (10^{-8} – 10^{-4} M, panel a) and IBMX (10^{-8} – 10^{-4} M, panel b) on **ACh**-induced contraction of isolated rat detrusor muscles at 10^{-4} M ACh. Panel (c) represents percentage change induced by the tested compounds on ACh-induced contraction. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from control ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (3). Effect of sildenafil (10^{-8} – 10^{-4} M, panel a) and IBMX (10^{-8} – 10^{-4} M, panel b) on electric field stimulation (**EFS**)-induced contraction of isolated rat detrusor muscles at frequency of 4 Hz. Panel (c) represents percentage change induced by the tested compounds on EFS-induced contraction. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from control ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (4). Effect of SNP (10^{-9} – 10^{-3} M, panel a) on **ACh**-induced contraction of isolated rat detrusor muscles at 10^{-4} M ACh. Panel (b) represents percentage change induced by SNP on ACh-induced contraction. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from control ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (5). Effect of SNP (10^{-9} – 10^{-3} M, panel a) on electric field stimulation (**EFS**)-induced contraction of isolated rat detrusor muscles at frequency of 4 Hz. Panel (b) represents percentage change induced by SNP on EFS-induced contraction. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from control ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (6). Effect of sildenafil (10^{-8} – 10^{-4} M) on electric field stimulation (**EFS**)-induced contraction of isolated rat detrusor muscles in absence and presence of L-NAME (10^{-4} M), N-PLA (3×10^{-5} M), MB (10^{-5} M) (panel a), or atropine ($0.1 \mu\text{M}$, panel b) at frequency of 4 Hz. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from sildenafil ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (7). Effect of sildenafil (10^{-7} M) on alprostadil (10^{-9} - 10^{-5} M)-induced potentiation of **ACh** (panel a) or electric field stimulation (**EFS**, panel b)-induced contraction of isolated rat detrusor muscles at concentration of 10^{-4} M ACh or frequency of 4 Hz. Responses are expressed as mean $\pm$ SEM. * denotes significant difference from control. # denotes significant difference from alprostadil ($p < 0.05$). Values between parentheses indicate the number of animals.

Figure (8). Proposed mechanism of interaction between alprostadil and sildenafil in isolated rat detrusor muscle (modified from Xia et al., ~~2004~~)[54]

AC= adenylyl cyclase, ATP= adenosine triphosphate, Ca^{2+} = calcium, cAMP= cyclic adenosine monophosphate, cGMP= cyclic guanosine monophosphate, DAG= diacylglycerol, EP= prostaglandin E receptor, $\text{G}_{q,s}$ = G-protein coupled receptors, GTP= guanosine triphosphate, IP3= inositol triphosphate, K^{+} = potassium, NO= nitric oxide, PKA= cAMP-dependent protein kinase, PKG= cGMP-dependent protein kinase, PLC= phospholipase C, sGC= soluble guanylyl cyclase

Figure (1)

Figure (2)

Figure (3)

Figure (4)

Figure (5)

Figure (6)

Figure (7)

Alprostadil

Detrusor Smooth Muscle

NO

Alprostadil

Ca^{2+}

EP₁

EP₃

G_q

G_q

PLC

DAG

IP₃

Contraction

Ca^{2+}

GTP

5'GMP

PKG

cGMP

sildenafil

IP

EP₂

EP₄

G_s

AC

ATP

cAMP

PKA

Relaxation

Ca^{2+}

K⁺