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A 6-NITRODOPAMINA É O PRINCIPAL MODULADOR ENDÓGENO DA CONTRATILIDADE DO DUCTO DEFERENTE HUMANO

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Dissertação apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Farmacologia.

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RESUMO

A contratilidade do ducto deferente é essencial na fase de emissão do processo ejaculatório. Controlada pelo sistema nervoso autônomo e predominantemente noradrenérgica. O ducto deferente é um modelo amplamente utilizado nos estudos da neurotransmissão simpática, especialmente o tecido de roedores, oferecendo vantagens práticas e econômicas.

O ducto deferente humano se origina na cauda do epidídimo, passa pelo canal inguinal e termina na base prostática, tem camada muscular espessa e é revestido por epitélio pseudoestratificado, com inervação se estendendo ao longo do ducto, formando um plexo denso, derivado de fibras simpáticas (T10-L2) e parassimpáticas (S2-S4). A fase de emissão envolve a transferência de fluido espermático pelo ducto deferente, desde os testículos até a uretra posterior.

Estudos mostram que a contração não é unicamente mediada pela noradrenalina, mas também por ATP e outros neurotransmissores. Pesquisas recentes destacam a 6-ND como modulador das contrações do ducto deferente de ratos.

A 6-ND tem ação contrátil de musculatura lisa em diversos tecidos humanos, sua liberação endógena é evidenciada pela presença em diferentes tecidos humanos. Esta nitrocatecolamina tem atividade biológica diversificada, incluindo a inibição da recaptação de noradrenalina e ação vasodilatadora específica, modula contrações cardíacas e potencializa a ação de outras catecolaminas.

Os antidepressivos tricíclicos são eficazes no tratamento da ejaculação precoce, sendo utilizados na prática clínica há décadas, mesmo embora, sejam classificados como parte do tratamento off-label. Essa classe de droga, com estrutura de três anéis aromáticos, inclui subtipos de amina terciária e secundária e era anteriormente a primeira linha de tratamento para depressão maior.

A vasectomia, forma permanente de esterilização masculina, não impacta a libido nem a função erétil. Estudos pós-vasectomia mostram que a inervação noradrenérgica do segmento distal do ducto diminui, o que pode afetar a maturação e transporte dos espermatozóides, influenciando no sucesso de uma eventual reversão do procedimento.

Palavras-chave: ejaculação; óxido nítrico; antidepressivos tricíclicos.

ABSTRACT

Vas deferens contractility is essential in the emissive phase of the ejaculatory process. It is controlled by the autonomic nervous system and is predominantly noradrenergic. The vas deferens model is widely used in studies of sympathetic neurotransmission, especially rodent tissue, which offers economical and practical advantages.

The human vas deferens originates at the epididymis tail, passes through the inguinal canal and ends at the base of the prostate. It has a thick muscle layer and is coated by pseudostratified epithelium, with innervation along the length of the duct forming a dense plexus, derived from sympathetic (T10-L2) and parasympathetic (S2-S4) fibers. The emissive phase involves the transfer of seminal fluid by the vas deferens, from the testicles to the posterior urethra.

Studies show that contraction is mediated not only by noradrenaline, but also by ATP and other neurotransmitters. Recent research highlights 6-ND as a modulator of vas deferens contractions in mice.

6-ND presents smooth muscle contractile action in various human tissues. Its endogenous liberation is evidenced by its presence in different human tissues. This nitrocatecholamine has diversified biological activity, including the inhibition of noradrenaline reuptake and specific vasodilating action, it modulates cardiac contractions and strengthens the action of other catecholamines.

Tricyclic antidepressants are effective in the treatment of premature ejaculation, having been used in clinical practice for decades, even though they are classified as part of off-label treatment. This drug class, with a structure of three aromatic rings, includes subtypes with tertiary and secondary amines and was previously the first-line treatment for major depressive disorder.

The vasectomy, a permanent birth control method for men, has no impact on libido or erectile function. Post-vasectomy studies show that the noradrenergic innervation of the duct's distal section decreases, which may affect the sperm's maturation and transport, affecting the success of an eventual procedure reversal.

Keywords: ejaculation; nitric oxide; tricyclic antidepressants.

LISTA DE ABREVIATURAS E SIGLAS

6-NA	6-nitroadrenalina
6-ND	6-nitrodopamina
Ach	acetilcolina
ATP	adenosina-tri-fosfato
EPSP	potenciais pós-sinápticos excitatórios
L-NAME	N-nitro-L-arginina metil éster
NA	noradrenalina
NO	óxido nítrico

SUMÁRIO

1. INTRODUÇÃO

A contratilidade do ducto deferente humano tem papel fundamental no processo ejaculatório, mais especificamente na fase de emissão, responsável pela transferência da secreção testicular até a uretra posterior masculina. Seu controle, assim como de sua secreção epitelial se dá através de inervação autonômica, sendo o controle motor, simpático, predominantemente noradrenérgico (1).

O ducto deferente é um bioensaio versátil e amplamente utilizado para o estudo da neurotransmissão autonômica simpática. Especialmente o tecido de roedores, com vantagens em relação a custo, acesso cirúrgico e preparação simples (2). Foi útil em pesquisas de fármacos adrenérgicos e vem sendo essencial na compreensão da co-transmissão simpática.

Modelos para o comando das contrações, vêm sendo propostos ao longo das últimas décadas evoluindo, desde co-transmissão de acetilcolina (Ach) e noradrenalina (NA) (3) a mediação pela NA (2), co-transmissão de adenosina-tri-fosfato (ATP) e NA (4), até às pesquisas mais recentes envolvendo a 6-Nitrodopamina (6-ND).

1.1- MORFOLOGIA DO DUCTO DEFERENTE HUMANO

O ducto deferente se origina na cauda do epidídimo, tortuoso em sua porção proximal por cerca de 3cm, emerge na pelve através do canal inguinal, acompanhando os vasos do cordão espermático. Na altura do anel inguinal interno se separa dos vasos testiculares e segue pela parede lateral pélvica até a base prostática, sua porção terminal, ampola, é capaz de armazenar espermatozóides. Seu suprimento sanguíneo vem da artéria vesiculodeferencial, ramo da artéria vesical superior. A drenagem venosa se dá através do plexo venoso pélvico, e a linfática, pelos gânglios ilíacos internos e externos.

Histologicamente temos uma parede espessa de musculatura lisa longitudinal externamente, e circular internamente, revestida por epitélio pseudoestratificado colunar com estereocílios não motores (1).

Ramos nervosos se estendem ao longo do ducto, na camada adventícia, em seu tecido conectivo, até atingirem, em segmentos menores, a camada muscular, formando um denso plexo nervoso, muitos destes, possuindo varicosidades. Originam-se de fibras simpáticas oriundas de T10 a L2 e fibras parassimpáticas de S2 a S4.

1.2- A CONTRAÇÃO DO DUCTO DEFERENTE HUMANO

A transferência do fluido espermático através do ducto deferente, com a propulsão de sua forte parede muscular é apenas parte do processo de emissão, primeira fase da ejaculação masculina. A emissão é um arco reflexo (T10-L2) iniciado com a ativação de receptores sensoriais localizados majoritariamente na glande (corpúsculos de Krause-Finger). As contrações se iniciam nos vasos retos testiculares, levando o sêmen até o epidídimo pelos ductos eferentes e em seguida ao ducto deferente. (5)

Tradicionalmente, inferia-se, que se tratando de uma neurotransmissão simpática adrenérgica, o estímulo contrátil se daria através da NA, seguindo-se o princípio de Dale, que sugeria que um nervo específico liberaria apenas um neurotransmissor em suas conexões sinápticas. Em 1961, Hukovic demonstrou em preparo de ducto deferente de porquinho da índia, com sua inervação simpática, o aumento da resposta contrátil após preparo do tecido com NA e a redução desta resposta após preparo do tecido com reserpina, um inibidor da captação de NA na fenda sináptica. A idéia de unidade na neurotransmissão de acordo com um nervo específico caiu com a descoberta de diversos outros neurotransmissores, e sua liberação possivelmente em conjunto por exocitose com a substância principal (6), sedimentando uma possível ação conjunta de neurotransmissores, visto o caráter bifásico da contração deste tecido (7).

Burnstock e Holman (1960 e 1961) demostraram que um estímulo nervoso único podia gerar potenciais pós-sinápticos excitatórios (EPSP) que se

somavam, levando à contração do músculo liso ao atingirem um limiar crítico de despolarização, e ainda, que antagonistas adrenérgicos não aboliam os EPSP, levando à observação de que ATP, atuando como um co-transmissor juntamente à NA, seria o responsável por estes EPSP (8). Contemporaneamente, outra pesquisa demonstrava que o ATP produziria contrações rápidas neste tecido e a NA, contrações lentas (9).

Com o avanço nos estudos com a 6-ND em outros tecidos e posteriormente em ducto deferente de rato, chegamos à observação de sua importante ação no ducto deferente humano, agindo como o principal modulador de suas contrações. Algumas evidências, como sua menor liberação após incubação tecidual com N-nitro-L-arginina metil éster (L-NAME), um inibidor da óxido nítrico (NO) sintetase, pareada com a promoção de contrações reduzidas após estímulo por campo elétrico, o antagonismo de sua ação contrátil por antidepressivos tricíclicos, se mostrando específico quando comparado à ação de outras catecolaminas, reforçam esta afirmação.

A demonstração de que antagonistas alfa adrenérgicos, observados clinicamente por levarem a efeitos colaterais relacionados a retardo ejaculatório ou anejaculação, bloqueiam as contrações mediadas pela 6-ND (10), e ainda que potencializa as contrações mediadas por outras catecolaminas (11), traz à tona ainda mais a importância e protagonismo desta molécula na modulação das contrações do ducto deferente humano.

1.3- A 6-NITRODOPAMINA

As nitrocatecolaminas se apresentam como moduladores não neurogênicos da contratilidade muscular autônoma, a observação de que o pré-tratamento de corpo cavernoso animal com L-NAME inibia o relaxamento ao mesmo tempo em que o pré-tratamento com tetrodotoxina, um inibidor dos canais de sódio voltagem dependentes, não o fazia (12); assim como a remoção endotelial de arco aórtico de serpente e o pré-tratamento deste tecido com alfa bloqueadores inibiam suas contrações, denota a ação e importância dos moduladores não neurogênicos, ou endoteliais.

A nitração de catecolaminas, in vitro, ocorre mediante processo simples, muito possivelmente reprodutível in vivo (13,14). A primeira identificação de nitrocatecolaminas endógenas se deu pela observação da redução na concentração de NA em cérebro de rato após a infusão do tecido com NO (15), usando os padrões da síntese de 6-nitroadrenalina (6-NA) in vitro em testes com cérebro de porco, identificou-se o mesmo padrão através de cromatografia líquida de alta pressão e caracterizou-se a 6-NA com espectrometria de massa (15).

A 6-ND, identificada através de cromatografia líquida associada a espectrometria de massa, in vitro, em artéria e veia umbilicais humanas (16) e artéria e veia poplíteas humanas (17), é uma 6-nitrocatecolamina com atividade biológica diversificada, como a inibição de recaptação neuronal de NA, atividade catecol-O-metiltransferase e atividade NO sintase (15). Nesses tecidos, a liberação basal de 6-ND foi reduzida com o tratamento com L-NAME e abolida com a remoção endotelial, além disso os neuromarcadores, proteína S-100 e calretinina, não foram encontrados nas túnicas íntima e média de arco aórtico de serpente e de tartaruga, reforçando sua origem não neurogênica (18).

A ação contrátil da 6-ND no músculo liso vascular diferencia-se da de outras catecolaminas, em artéria e veia umbilicais humanas, o pré-tratamento do tecido com 6-ND levou à inibição das contrações mediadas por dopamina, sem afetar as mediadas por noradrenalina ou adrenalina (16), nesses tecidos a dopamina causa contrações através de receptores dopaminérgicos D2, sendo estas antagonizadas por haloperidol, um antipsicótico antagonista específico dos receptores D2 (19). Esses achados sugerem uma ação vasodilatadora da 6-ND, via antagonismo D2, e trazem uma via alternativa para o NO como agente relaxante vascular, uma vez que o relaxamento do músculo liso é atribuído à geração de GMP cíclico através da guanilato ciclase (20) e o relaxamento mediado pela 6-ND não foi afetado pela pré incubação com o inibidor da ativação de guanilato ciclase (ODQ) (16).

Em tecido cardíaco de rato a 6-ND apresentou, assim como a dopamina, noradrenalina e adrenalina, um efeito cronotrópico positivo, porém 10000 vezes mais potente que a primeira e 100 vezes mais potente que as outras. Outra característica importante é sua capacidade de potencialização da ação contrátil

e cronotrópica de outras catecolaminas neste tecido (21) fato este, também observado em testes de contratilidade em ducto deferente de rato (22). Uma alternativa para o controle da reatividade vascular e débito cardíaco seria uma possível liberação de 6-ND pela musculatura estriada ao ser contraída, levando a um feedback positivo de resposta cardíaca para a manutenção da atividade muscular (23).

1.4- OS ANTIDEPRESSIVOS TRICÍCLICOS

O tratamento farmacológico da ejaculação precoce é até atualmente restrito a poucas drogas, em se tratando de medicamentos que passaram por estudos clássicos visando o seu registro oficial, a Dapoxetina, um inibidor da recaptação de serotonina de curta ação usada sob-demanda é a única opção de tratamento oral (24) embora as prescrições mais comuns sejam as consideradas off-label, dentre elas destacamos a clomipramina, um antidepressivo tricíclico com resultados no tratamento da ejaculação precoce de incremento no IELT (tempo de latência para ejaculação intra-vaginal) de 512% (25), e usada na prática clínica desde os anos 70. Associado aos achados de que os antidepressivos tricíclicos atuam como antagonistas seletivos dos receptores de 6-ND em ducto deferente de rato (22), foram selecionados para os testes em ducto deferente humano neste estudo os antidepressivos tricíclicos: desipramina e amitriptilina; associado a estes, a carbamazepina, um antipsicótico, bloqueador de canais de sódio voltagem dependente, com estrutura molecular tricíclica e ação farmacológica semelhante à dos primeiros.

Esta classe de drogas, nomeada a partir de sua estrutura química, com três anéis aromáticos ligados a uma cadeia lateral, são divididos em 2 grupos: 1) subtipo amina terciária (amitriptilina, dotiepina, imipramina e clomipramina) e 2) subtipo amina secundária (nortriptilina, protriptilina e desipramina); sendo por vezes, as drogas do grupo 1, metabólitos de drogas do grupo 2 (26), já foram, nos anos 50 e 60, primeira linha de tratamento de depressão maior, sendo aos poucos, substituídas nesta terapêutica, por drogas com menos efeitos adversos e menor potencial de letalidade em overdoses.

Sua farmacocinética é caracterizada por ampla primeira passagem pré sistêmica, grande volume de distribuição e extensa ligação proteica, com meia-vida de aproximadamente 1 dia, tem inativação hepática via citocromo p450, com desmetilação dos fármacos do grupo amina terciários para seus metabólitos secundários, que sofem hidroxilação e então glicuronidação para sua excreção urinária (27).

Os inibidores da recaptação de serotonina, drogas tidas como de terceira geração dos antidepressivos, atingiram sucesso clínico e comercial por terem dupla ação, como inibidores da recaptação da serotonina e da noradrenalina, propriedade esta, compartilhada por alguns antidepressivos tricíclicos.

Na tabela a seguir (28) comparam-se os Ki (constante de equilíbrio de dissociação) de diferentes antidepressivos tricíclicos, em relação a diferentes receptores pré e pós-sinápticos.

Table 3 Receptor profile, K_i (nmol/l), of TCAs and comparator drugs: uptake inhibition and receptor antagonism (HCR data)

Drug	*Reuptake inhibition*		*Post-synaptic receptor antagonism*			
	5-HT	*NA*	*H1*	α_1	*Musc*	*5-HT$_{2A}$*
Mirtazapine*	>10 000	4600	0.14	500	670	16
Mianserin*	>4000	71	0.40	34	820	7
Doxepin	68	29.5	0.24	24	83	25
Amitriptyline	20	50	1	27	18	29
Imipramine	7	60	40	32	46	80
Clomipramine	0.14	54	15	32	25	35
Nortriptyline	100	10	6.3	55	37	44
Dothiepin	78	70	4	400	38	260
Desipramine*	18	0.83	110	100	100	280
Reboxetine*	58	7.2	310	>1000	>1000	>1000

Abbreviations: HCR, human cloned receptor.
Smaller K_i values represent greater potency. Note: where values are available from different laboratories and different experiments, affinities can vary by about one order of magnitude; mid-range values are given (Table 2a and b gives ranges).
Receptors: H1, Histamine type 1; Musc, acetylcholine muscarinic; α_1, α_1 adrenoceptor. Note that no HCR data are known for lofepramine.
All data have been extracted from PDSP K_i database, http://pdsp.med.unc.edu/pdsp.php (except *Richelson, 2001).

1.5- VASECTOMIA E A INERVAÇÃO DO DUCTO DEFERENTE HUMANO

A vasectomia, método cirúrgico de ligadura bilateral do ducto deferente, é uma forma permanente de esterilização, confiável e de baixo custo, menos

invasiva que a ligadura de tubária feminina. Este procedimento não afeta a libido masculina, nem altera os níveis de testosterona sanguíneos ou a função erétil, resulta em um menor volume ejaculado, embora raramente perceptível, uma vez que o volume da secreção testicular corresponde a aproximadamente 5% do total ejaculado.

Com relação aos efeitos da vasectomia no ducto remanescente, há um estudo em humanos (29) em que foram testados usando neurohistoquímica, amostras cirúrgicas dos cotos proximais e distais em procedimentos de vasovasostomia, realizados entre 1 a 15 anos após a vasectomia original. Em relação aos tecidos controles e dos cotos proximais (uretrais), os tecidos de cotos distais (testiculares), tiveram redução da inervação noradrenérgica da camada muscular. Este achado condiz com a idéia de que as fibras nervosas no ducto deferente se estendem no sentido uretral-testicular. Após a vasectomia pode haver reinervação parcial da camada muscular do segmento distal a partir dos nervos perivascularespoupados no procedimento, no entanto a inervação subepitelial não é capaz de regeneração, mantendo o epitélio da porção testicular denervado. Esta denervação pode ter efeitos no sucesso de uma eventual vasovasostomia, pois a maturação, motilidade e transporte dos espermatozóides podem ser comprometidos (30).

2- JUSTIFICATIVA E HIPÓTESE

No canal deferente de ratos, observou-se que os antidepressivos tricíclicos e os antagonistas dos receptores alfa-1 adrenérgicos antagonizaram o efeito contrátil induzido pela 6-ND. Portanto, neste trabalho, hipotetizamos que essas classes de medicamentos atuem de maneira semelhante no canal deferente humano e que, possivelmente, atuem preferencialmente no receptor da 6-ND e não nos receptores das catecolaminas clássicas.

3. RESULTADOS

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ORIGINAL ARTICLE

6-nitrodopamine is a major endogenous modulator of human vas deferens contractility

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Abstract

Background: Rat isolated *vas deferens* releases 6-nitrodopamine (6-ND), and the spasmogenic activity of this novel catecholamine is significantly reduced by tricyclic compounds such as amitriptyline, desipramine, and carbamazepine and by antagonists of the α_1-adrenergic receptors such as doxazosin, tamsulosin, and prazosin.

Objectives: To investigate the liberation of 6-ND by human epididymal vas deferens (HEVDs) and its pharmacological actions.

Methods: The in vitro liberation of 6-ND, dopamine, noradrenaline, and adrenaline from human vas deferens was evaluated by LC-MS/MS. The contractile effect of the catecholamines in HEVDs was investigated in vitro. The action of tricyclic antidepressants was evaluated on the spasmogenic activity ellicited by the catecholamines and by the electric-field stimulation (EFS). The tissue was also incubated with the inhibitor of nitric oxide (NO) synthase L-NAME and the release of catecholamines and the contractile response to EFS were assessed.

Results: 6-ND is the major catecholamine released from human vas deferens and its synthesis/release is inhibited by NO inhibition. The spasmogenic activity elicited by EFS in the human vas deferens was blocked by tricyclic antidepressants only at concentrations that selectively antagonize 6-ND induced contractions of the human vas deferens, without affecting the spasmogenic activity induced by dopamine, noradrenaline, and adrenaline in this tissue. Incubation of the vas deferens with L-NAME reduced both the 6-ND release and the contractions induced by EFS.

Discussion and conclusion: 6-ND should be considered a major endogenous modulator of human vas deferens contractility and possibly plays a pivotal role in the emission process of ejaculation. It offers a novel and shared mechanism of action for tricyclic antidepressants and α_1-adrenergic receptor antagonists.

KEYWORDS
ejaculation, nitric oxide, tricyclic antidepressants

1 | INTRODUCTION

Emission occurs following a sympathetic spinal cord reflex (T10-L2) stimuli following the activation of sensory receptors located in the glans (Krause-Finger corpuscles). The physiological process of the male ejaculation initiates with the increase of the contractile activity of the duct that extends from the rete testis to the epididymis (*vasa efferentia*).[1] Contractions of the vas deferens also have an important role in the propulsion of the spermatozoa and occur near-simultaneously with the contractions of the seminal glands.[2] 6-nitrodopamine (6-ND) is a new catecholamine that has been recently shown to be released from rat isolated vas deferens[3] and atria[4] and from human umbilical cord vessels.[5] The synthesis/release of 6-nitrodopamine is reduced (but not abolished) when these tissues are incubated with the nitric oxide (NO) synthase inhibitor L-NAME. 6-nitrodopamine can ben synthesized in vitro by incubation dopamine with nitric acid,[6] which generates NO or peroxynitrite.[6] Thus, nitrosation of dopamine is apparently an essential step in the formation of 6-nitrodopamine. Whether this process in vivo is a chemical, or enzymatic process is yet to be determined. Since 6-nitrodopamine is a potent vasodilator,[5,7] the formation of this novel catecholamine rather than stimulation of soluble guanlylate cyclase[8] may be an important mechanism of action for NO. The contractions caused by 6-ND in the rat isolated vas deferens are selectively antagonized by tricyclic antidepressants such as amitriptyline, desipramine and clomipramine[3] and by α_1-adrenergic receptor blockers such as doxazosin, tamsulosin and silodosin.[9]

The clinical use of tricyclic antidepressants has been associated with delayed ejaculation,[10] and the tricyclic antidepressant clomipramine has therapeutic indication in the control of premature ejaculation in man.[11–13] Lower urinary tract symptoms (LUTSs) are generally treated with α_1-adrenergic receptor blockers, but reduced ejaculate volume, absence of ejaculate, and retrograde ejaculation are observed in 4%–11% of patients.[14–15]

Since both tricyclic antidepressants and α_1-adrenergic receptor antagonists are potent and selective 6-ND receptor antagonists in the rat vas deferens, these findings open the exciting possibility that these drugs could have similar mechanism in the human vas deferens. Thus, we have investigated whether 6-ND is released by human vas deferens and its actions in this tissue.

2 | MATERIALS AND METHODS

2.1 | Study participants

Participants who underwent vasectomy surgery from Hospital e Maternidade Salvalus were asked to sign an informed consent approved by the University of Sao Paulo Institutional Review Board (protocol number 4.468.508). The human epididymal vas deferens (HEVDs) were obtained from 96 participants aged 28–53 years. The vasectomy[16] was performed under local anaesthesia, and the excised segment (2-cm long, taken approximately at 9 cm from the cauda epididymis) is illustrated in Figure 1.

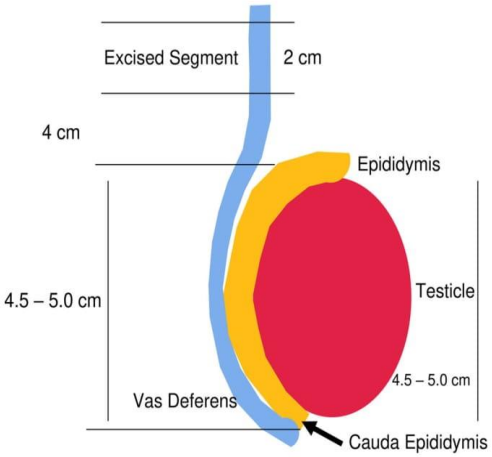

FIGURE 1 The vasectomy illustration

2.2 | Catecholamine basal release

Two HEVD strips (15 mm length) were suspended in a 5-ml glass chamber containing warmed (37°C) and oxygenated (95% O_2 / 5% CO_2) Krebs-Henseleit's solution (KHS) containing ascorbic acid (3 mM) to prevent catecholamine oxidation.[17] The NO synthase inhibitor L-NAME (100 μM) was incubated for 30 min. Samples (2 ml) of the KHS were transferred to amber Eppendorf vials and frozen at −20°C until analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.3 | Determination of catecholamine concentrations by mass spectrometry

The method employed for 6-ND quantification[18] was modified to allow the measurement of the four catecholamines in a single chromatographic run. Briefly, the extraction of the catecholamines from KHS (1 ml) was performed by solid phase extraction. To 1 ml of KHS was added 50 μl (100 ng/ml) of the deuterated catecholamines used as internal standards, and the samples were homogenized for 10 s. The Strata-X 33 mm Polymeric Reversed Solid Phase Extraction (SPE) cartridges were prewashed with MeOH (1 ml) followed by deionized H_2O (2 ml). After sample introduction into the cartridge, the cartridge was subsequently washed three times with deionized H_2O. The catecholamines were then eluted with 900 μl MeOH/H_2O (90/10, v/v) with formic acid (0.1%). The eluate was evaporated under N_2 flow at 50°C. The residue was dissolved with 100 μl of acetonitrile/H_2O (50/50, v/v) with 0.1% formic acid and transferred to vials ready for injection into the mobile phase (75% A composed of deionized H_2O with 0.1% formic acid (v/v) and 25% B composed of acetonitrile/H_2O (90/10, v/v) with 0.1% formic acid. The mobile phase perfused an LC ADVp

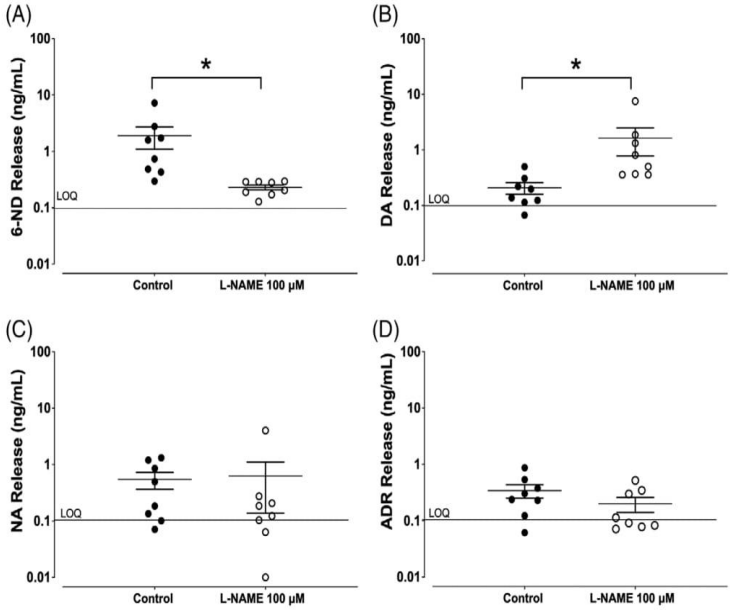

FIGURE 2 Basal release of 6-nitrodopamine (6-ND) (A), dopamine (B), noradrenaline (C), and adrenaline (D) were detected in the human epididymal vas deferens (HEVD). The basal release was measured by LC-MS/MS following a 30 min period incubation in Krebs-Henseleit's solution in the absence and the presence of L-NAME (100 μM). *$p < 0.05$ control versus L-NAME

Liquid Chromatograph Shimadzu System coupled to a Shimadzu 8060 triple quadrupole mass spectrometer operating in ESP^+ mode at 350 μl/min. The dissolved residues were injected by a SIL-30AC autoinjector, at a temperature of 8°C. The transitions monitored by electrospray multiple reaction monitoring, injection volume, run-time, and limit of quantitation were described elsewhere.[18] The linearity was given in a range of 0.1–20 ng/ml, and the method validation was carried following the Food and Drug Adminisration (FDA) guidelines for bioanalytical methods.[19]

2.4 | HEVD functional assays

Each HEVD strip was suspended vertically between metal hooks in 10-ml glass chamber containing heated (37°C) and oxygenated (95%O_2: 5%CO_2) KHS. Tissues were maintained at 10 mN, and the isometric tension was recorded by a PowerLab system. After a stabilization interval (45-min), the HEVD strips were initially contracted with potassium chloride (KCl, 80 mM) to assess the tissue viability. After KCl removal and return to the baseline (15 min approximately), cumulative concentration-response curves to 6-ND were performed in control HEVD strips and in tissues preincubated with amitriptyline (30-300 nM, 30 min), desipramine (30-300 nM, 30 min) and/or carbamazepine (30–300 nM, 30 min).

Cumulative concentration-response curves to dopamine, noradrenaline, and adrenaline were performed in HEVD pre-incubated or not with amitriptyline (100 nM, 30 min), desipramine (100 nM, 30 min), and carbamazepine (100 nM, 30 min).

2.5 | Electric-field stimulation in HEVD preparations

The HEVD strips were submitted to electric-field stimulation (EFS) (60 V for 20 sec, at 2–32 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay), using a Grass S88 stimulator. The EFS-induced HEVD contractions were conducted in tissues control and tissues preincubated with amitriptyline (100 nM, 30 min), desipramine (100 nM, 30 min), and carbamazepine (100 nM, 30 min).

2.6 | Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 9.0, San Diego, California, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10c / 10x)^n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The values of pEC_{50} data represent the mean ± standard error of the mean of *n* experiments. Values of E_{max} were expressed in mN. One strip was used as the control response, and the other strip was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and

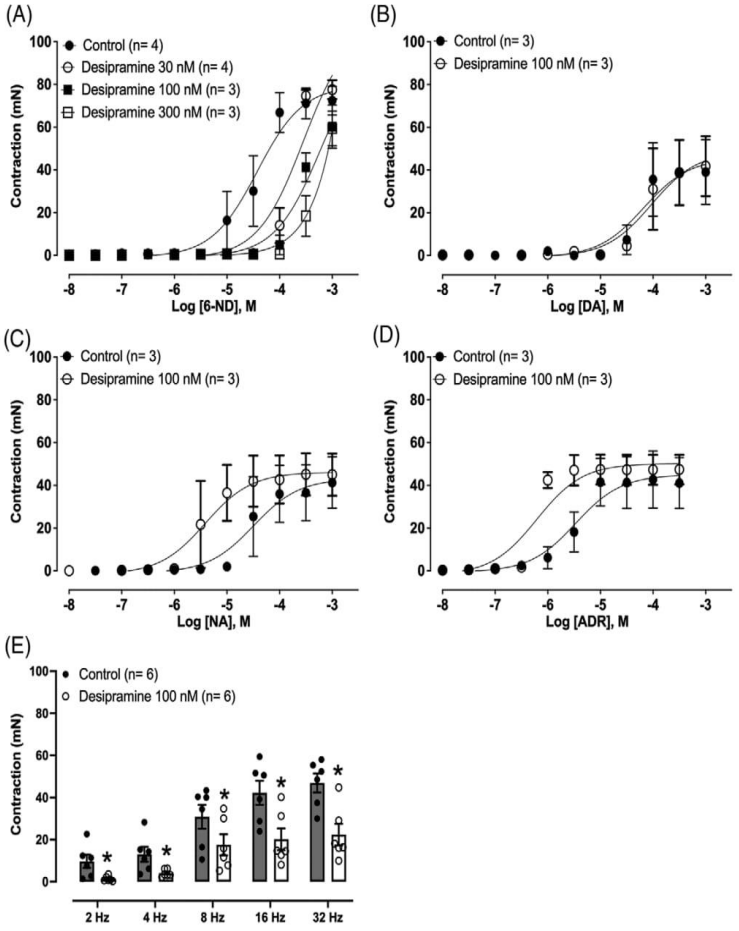

FIGURE 3 Effect of desipramine in the human epididymal vas deferens (HEVD). Desipramine (30-300 nM) causes a significant concentration-dependent rightward shifts on the concentration-response curves to 6-nitrodopamine (6-ND) (A). Desipramine (100 nM) had no effect on the contractions induced by dopamine (B) but caused a significant leftward shift on the concentration-response curves to noradrenaline (C) and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Desipramine (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). $^*p < 0.05$. *n* means the number of the HEVD.

the differences between groups. In addition, standard analysis of variance (ANOVA), followed by the Newman–Keuls post-test, were used when more than two groups were involved. A *p* value of less than 0.05 was considered statistically significant. For 6-ND, the pA_2 values of the antagonists were calculated from the intercept on the concentration axis and by application of the equation; pA_2 = log (antagonist concentration) –log (CR-1) -log (antagonist concentration).[20]

3 | RESULTS

3.1 | Basal release of catecholamines

6-nitrodopamine (Figure 2A), dopamine (Figure 2B), noradrenaline (Figure 2C), and adrenaline (Figure 2D) were detected by LC-MS/MS in HEVD strips. Incubation (30 min) of the HEVD strips with L-NAME (100 μM) significantly reduced the 6-ND levels (1.91 ± 0.82 and 0.23 ± 0.03 ng/ml, for control and L-NAME respectively; Figure 2A), and an equivalent increase in basal dopamine release was observed (0.20 ± 0.05 and 1.63 ± 0.86 ng/ml, for control and L-NAME, respectively; Figure 2B). Incubation (30 min) of the HEVD strips with L-NAME (100 μM) had no effect in the levels of noradrenaline (Figure 2C) and adrenaline (Figure 2D).

3.2 | Effect of desipramine

Pretreatment (30 min) with desipramine (30-300 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.48 ± 0.21; Figure 3A; $p = 0.0015$) with a pA_2 value of 9.46 ± 0.07($n = 4$). Desipramine (100 nM) did not affect the HEVD contractions induced by dopamine (Figure 3B; pEC_{50} 4.18 ± 0.16

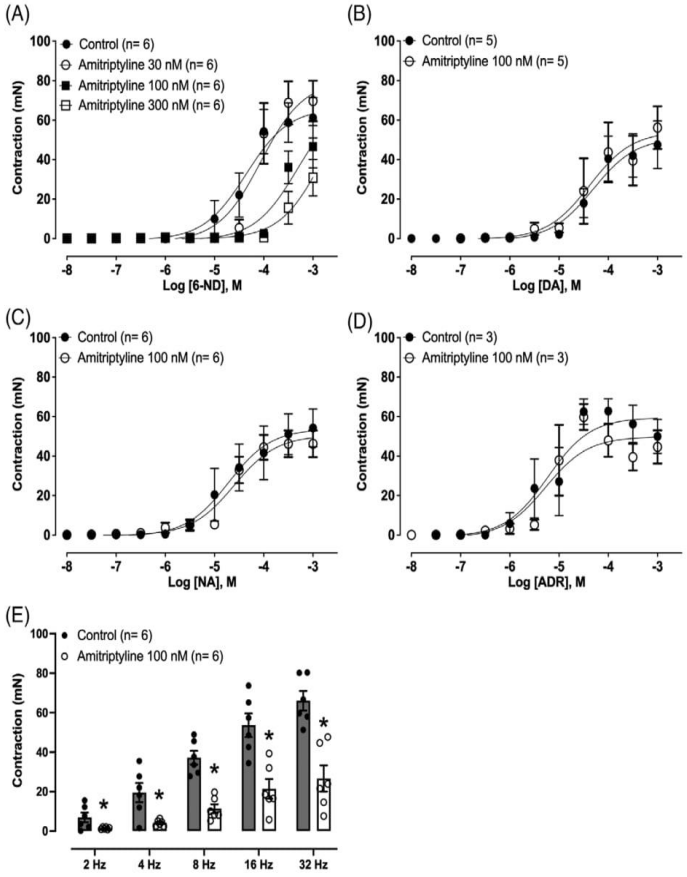

FIGURE 4 Effect of amitriptyline in the human epididymal vas deferens (HEVD). Amitriptyline (100 nM) produced concentration-dependent rightward shifts on the concentrationresponse curves to 6-ND (A) and at 300 nM, amitriptyline markedly reduced the maximal responses to 6-nitrodopamine (6-ND). Amitriptyline (100 nM) had no effect on the contractions induced by dopamine, noradrenaline (C), and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Amitriptyline (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). $^*p < 0.05$. *n* means the number of the HEVD.

and 4.03 ± 0.16, for control and treated; respectively; $p = 0.4795$) but caused significant leftward shifts on the concentration-response curves to noradrenaline (Figure 3C; pEC_{50} 4.49 ± 0.16 and 5.41 ± 0.15, for control and treated, respectively; $p = 0.014$) and adrenaline (Figure 3D; pEC_{50} 5.47 ± 0.13 and 6.21 ± 0.08, for control and treated, respectively; $p = 0.0095$).

Desipramine (100 nM) significantly antagonized the HEVD contractions induced by EFS (2–32 Hz; Figure 3E).

3.3 | Effect of amitriptyline

Pretreatment (30 min) with amitriptyline (100 and 100 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.33 ± 0.14; Figure 4A; $p = 0.0031$) with a pA_2 value of 8.86 ± 0.09($n = 6$). However, at 300 nM, amitriptyline markedly reduced the maximal responses to 6-ND (Emax; 61.17 ± 7.54 and 30.87 ± 12.60 mN, for control and amitriptyline 300 nM, respectively; $p = 0.0026$), making the pEC_{50} calculation inaccurate. Amitriptyline (100 nM) did not alter the HEVD contractions induced by dopamine (Figure 4B; pEC_{50} 4.31 ± 0.08 and 4.38 ± 0.11, for control and treated; $p = 0.6081$), noradrenaline (Figure 4C; pEC_{50} 4.72 ± 0.09 and 4.60 ± 0.05, for control and treated; $p = 0.2925$) and adrenaline (Figure 4D; pEC_{50} 5.23 ± 0.11 and 5.27 ± 0.12, for control and treated; $p = 0.7911$).

Amitriptyline (100 nM) significantly reduced the HEVD contractions induced by EFS (2–32 Hz; Figure 4E).

3.4 | Effect of carbamazepine

Pretreatment (30 min) with carbamazepine (100 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.36 ± 0.09; Figure 5A; $p = 0.0148$) with a pA_2 value of

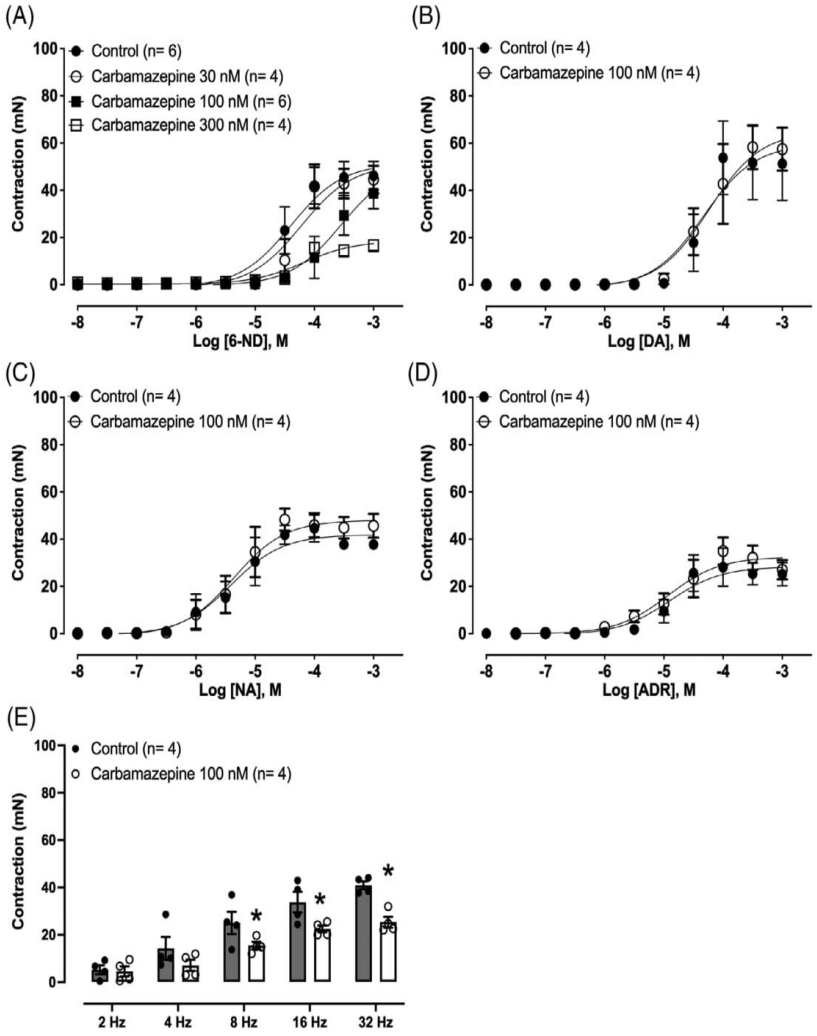

FIGURE 5 Effect of carbamazepine in the human epididymal vas deferens (HEVD). Carbamazepine (100 nM) produced concentration-dependent rightward shifts on the concentration-response curves to 6-nitrodopamine (6-ND) (A) and at 300 nM, carbamazepine markedly reduced the maximal responses to 6-ND. Carbamazepine (100 nM) had no effect on the contractions induced by dopamine, noradrenaline (C), and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Carbamazepine (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). $^*p < 0.05$. *n* means the number of the HEVD.

8.77 ± 0.067(n = 6). However, at 300 nM, carbamazepine markedly reduced the maximal responses to 6-ND (Emax; 46.1 ± 6.1 and 16.7 ± 2.72 mN, for control and carbamazepine 300 nM, respectively; $p = 0.0028$), making the pEC_{50} calculation inaccurate. Carbamazepine (100 nM) did not affect the HEVD contractions induced by dopamine (Figure 5B; pEC_{50} 4.33 ± 0.10 and 4.23 ± 0.08, for control and treated; $p = 0.4883$), noradrenaline (Figure 5C; pEC_{50} 5.41 ± 0.08 and 5.36 ± 0.07, for control and treated; $p = 0.6912$) and adrenaline (Figure 5D; pEC_{50} 4.90 ± 0.09 and 4.92 ± 0.08, for control and treated; $p = 0.8309$).

Carbamazepine (100 nM) significantly reduced the HEVD contractions induced by EFS (2–32 Hz; Figure 5E).

3.5 | Effect of L-NAME

EFS caused frequency-dependent contractions of the HEVDs. Pretreatment (30 min) with L-NAME (100 μM) significantly inhibited the contractions of the HEVD induced by EFS (2–32 Hz; Figure 6).

4 | DISCUSSION

This is the first demonstration that 6-ND is the major catecholamine released from human vas deferens. The release of 6-ND was reduced by pretreatment of the vas deferens with L-NAME, indicating that the

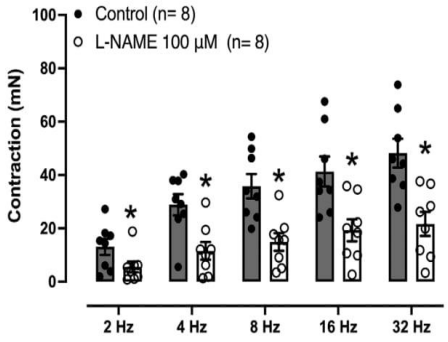

FIGURE 6 Electric-field stimulation (EFS) caused frequency-dependent contractions of the human epididymal vas deferens (HEVD). The pretreatment with L-NAME (100 μM) caused a significant reduction in the contractions induced by EFS in HEVD. $^*p < 0.05$. *n* means the number of the HEVD.

synthesis/release of 6-ND is coupled to NO synthesis. Pretreatment of human umbilical cord vessels[5] and rat vas deferens[3] with L-NAME is accompanied by decrease of 6-ND synthesis/release. Chronic treatment of rats with L-NAME also decreases the 6-ND release by vas deferens.[3] However, the effect of L-NAME pretreatment in the human vas deferens has two distinct characteristics of the rat, namely, in the rat vas deferens, although incubation with L-NAME decreased the basal release of 6-ND, it did not affect the contractions induced by EFS, an indication that 6-ND is probably stocked in vesicles in the animal species. Another interesting difference is that in contrast to the rat vas deferens, inhibition of 6-ND in human vas deferens is accompanied by an equivalent increase of dopamine levels. This difference cannot be attributed to species differences (man and rat), since the inhibition of 6-ND synthesis/release by L-NAME in human cord vessels was not accompanied by increase in dopamine levels. Thus, EFS in human vas deferens must stimulate 6-ND synthesis. The finding that L-NAME pretreatment does not abolish 6-ND synthesis/release could be due to a synthetic pathway independent of NO synthase activation. For instance, 6-ND and 6-hydroxydopamine can be formed by nitrite- and peroxide-dependent oxidation pathways of dopamine.[6] Another possibility is the existence of another enzymatic pathway for the synthesis of 6-ND independent of NO synthase.

Tricyclic antidepressants increase both serotonin and noradrenaline levels in the synaptic cleft[21,22] due to inhibition of the reuptake of these monoamines following the binding of the tricyclic antidepressants to serotonin reuptake transporter (SERT)[23] and norepinephrine transporter (NET).[24] Based on this proposed mechanism of action, it is surprising that the use of tricyclic antidepressants is associated with delayed ejaculation, since the increase in noradrenaline levels should result in increased contractility of the vas deferens. Amitriptyline, nortriptyline and imipramine bind to α_{1A}-, α_{1B}-, and α_{1D}- adrenergic receptors in HEK-293 cells expressing human α_1- receptor subtypes.[25] They also bind to α_1-adrenergic native receptors in rat vas deferens; however, they were much less potent in the competition for the specific binding of [^{3}H]-prazosin,[25] indicating that this antagonism is unlikely to overcome the increase of noradrenaline resulting from NET inhibition. Amitriptyline (100 nM–10 μM) inhibited neurogenic induced contractions in human vas deferens, although at 10 μM it also attenuated the contractions elicited by KCl, indicating a nonspecific mechanism at higher concentration.[26] At 100 nM, we did not find inhibition by amitriptyline of noradrenaline-induced contractions of the human vas deferens, indicating that the inhibition of the neurogenic induced contractions was not due to blockade of the α_1-adrenergic receptors in this tissue.

As observed in the rat vas deferens[3] dopamine and 6-ND have similar pEC_{50} values in the human vas deferens (4.28 ± 0.12 and 4.11 ± 0.10 for dopamine and 6-ND, respectively). Yet, L-NAME pretreatment caused a significant inhibition of EFS-induced contractions, although the inhibition of 6-ND synthesis/release was accompanied by a similar increase in dopamine synthesis/release. Since 6-ND and dopamine cause vas deferens contraction by acting in different receptors, one possible explanation for this apparent paradox could be a distinct location of these receptors. Purification and sequence of the 6-ND receptor should provide further information on the interactions of this novel mediator with the classical catecholamines in human vas deferens.

EFS causes NO release in human corpus cavernosum.[27] Since NO promotes smooth muscle relaxation,[28] the finding that L-NAME inhibits EFS-induced HEVD contractions demonstrates that endogenously produced 6-ND, and not NO, acts as a direct mediator of HEVD contractility.

5 | CONCLUSION

The identification of 6-ND as a novel mediator of HEVD contractility provides a new insight on the physiological process of the male ejaculation and presents a promising therapeutic target for ejaculatory disorders.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, investigation, methodology, writing – original draft, writing – review and editing: JBJ. *Investigation and methodology*: WPSF, ACA. RC, MOM, and MEAM. *Methodology*: AF. *Methodology and supervision*: FZM. *Funding acquisition, methodology, supervision, visualization, writing – original draft, writing – review and editing*: EA. *Conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, writing – review and editing, investigation, visualization, writing – original draft*: GDN.

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4. CONCLUSÃO

A definição da 6-ND como um novo mediador da contratilidade do HEVD fornece um novo ponto de vista do processo fisiológico ejaculatório masculino e apresenta um alvo promissor na terapêutica dos distúrbios ejaculatórios.

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