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RESEARCH ARTICLE

Vitamin D deficiency induces erectile dysfunction: Role of superoxide and Slpi

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Background and Purpose: Epidemiological studies suggest a relationship between vitamin D deficiency and erectile dysfunction (ED). We hypothesized that vitamin D deficiency or vitamin D receptor (VDR) knockout causes ED and analysed the underlying molecular mechanisms.

Experimental Approach: Erectile function was assessed in vivo in anaesthetized male mice or rats by evaluating intracavernosal pressure (ICP) and in vitro in male $Vdr^{-/-}$ mice, and rat or human isolated corpora cavernosa (CCs) mounted in a myograph. Bulk RNA-sequencing (RNA-seq) transcriptomic analysis was performed in rat CCs. Vitamin D deficiency was induced in rats fed a vitamin D-free diet for 5 months.

Key Results: CCs from human donors with low plasma vitamin D exhibited reduced nitric oxide (NO)-dependent erectile function. This ED was also reproduced in vitamin D-deficient rats and VDR knockout mice, in vivo and ex vivo, and is associated with penile fibrosis and reduced response to the phosphodiesterase 5 inhibitor (PDE5i) sildenafil. CCs from deficient rats show increased superoxide levels, and their impaired erectile function was restored by superoxide scavengers. Transcriptomic analysis, real-time polymerase chain reaction (RT-PCR) and Western blot showed down-regulated secretory leukocyte protease inhibitor (*Slpi*). Moreover, recombinant SLPI prevented superoxide-induced ED, while *Slpi* gene silencing led to reduced erectile function in a superoxide-dependent manner.

Abbreviations: CCs, corpora cavernosa; DEA-NO, diethylamine NONOate; DHE, dihydroethidium; ED, erectile dysfunction; EFS, electrical field stimulation; ICP, intracavernosal pressure; PDE5i, phosphodiesterase 5 inhibitor; PEGSOD, PEGylated superoxide dismutase; sGC, soluble guanylyl cyclase; SLPI, secretory leukocyte protease inhibitor; *Slpi*, secretory leukocyte protease inhibitor gene; TEMPOL, 4-hydroxy-TEMPO; U46619, (5*Z*)-7-{(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxyoct-1-en-1-yl]-2-oxabicyclo[2.2.1]heptan-5-yl}hept-5-enoic acid; VDR, vitamin D receptor; *Vdr*, vitamin D receptor gene; VDRE, vitamin D response element.

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Conclusion and Implications: Vitamin D deficiency or VDR knockout reduces erectile function. We suggest that this effect is mediated by increased superoxide levels and down-regulation of SLPI. Vitamin D deficiency might be an aetiological factor for vascular ED and for the therapeutic failure of PDE5i.

KEYWORDS
corpora cavernosa, erectile dysfunction, nitric oxide, SLPI, superoxide, vitamin D deficiency

1 | INTRODUCTION

Erectile dysfunction (ED) is characterized by the inability to achieve or maintain an erection of the penis during sexual activity (Shamloul & Ghanem, 2013). It is a highly prevalent condition, affecting 30% of European males aged 40–79 years and 52% of US males aged 40–70 years (Yafi et al., 2016). Moreover, it adversely impacts the quality of life and is considered a sentinel marker for poor general health (Kessler et al., 2019). In fact, ED has independent predictive value for future myocardial infarction and stroke (Inman et al., 2009). Obesity, metabolic diseases and diabetes are well-recognized risk factors for sexual dysfunction (Shamloul & Ghanem, 2013). Impaired bioactivity of nitric oxide (NO) released by nerve and endothelial cells in the corpora cavernosa (CCs) of the penis is a major pathogenic mechanism in ED (Mitidieri et al., 2020). Contemporary treatment algorithms for ED involve the use of **phosphodiesterase 5 inhibitors (PDE5is)** as first-choice agents to potentiate the NO/cyclic guanylyl cyclase (cGMP) pathway (Shamloul & Ghanem, 2013). It is estimated that 30% of patients with ED are non-responders to PDE5i, and intracavernosal injection of prostaglandins or other vasoactive agents remains as an alternative therapy (Park et al., 2013).

Vitamin D, mainly synthesized in skin after UVB exposure, has physiological functions beyond calcium and phosphorus homeostasis, including the regulation of cellular growth, intracellular metabolism, and innate and adaptive immunity (Ramasamy & Vitamin, 2020). Vitamin D status is an important health issue, as more than half of the world population exhibits vitamin D deficiency (Roth et al., 2018), usually defined as 25-hydroxyvitamin D plasma levels below 50 nM (equal to 20 ng·ml^{-1}). Vitamin D exerts multiple protective effects on vascular function (Anilkumar et al., 2024). However, there are many controversies in the field, and there is a specific need to analyse the role of severe vitamin D deficiency (Gallagher & Rosen, 2023). Epidemiological studies have reported a higher prevalence of vitamin D deficiency in ED patients, an association between the severity of ED and 25-hydroxyvitamin D plasma levels and an improved response to PDE5i after vitamin D replacement (Canguven & Al Malki, 2021; Demirci et al., 2021; Horsanali et al., 2020). However, the cause–effect relationship is unclear, and the potential mechanisms are unknown. Recently, a large randomized study has failed to demonstrate positive effects of vitamin D supplements versus placebo on ED. However, the patients in the placebo arm of the study had average 25-hydroxyvitamin D levels well above the cut-off of vitamin deficiency (75 [SD 20] nM) (Duarte Romero et al., 2024).

Herein, we questioned whether isolated human CCs from organ donors with vitamin D deficiency exhibit impaired erectile function

What is already known

- Epidemiological studies have reported a higher prevalence of vitamin D deficiency in erectile dysfunction patients.

What does this study add

- Isolated human corpora cavernosa from organ donors with vitamin D deficiency exhibit impaired erectile function.
- Erectile dysfunction is reproduced in vitamin D deficiency rats and vitamin D receptor knockout mice.

What is the clinical significance

- Restoring vitamin D in erectile dysfunction patients might improve sexual performance and efficacy of therapies.

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and if this is reproduced ex vivo and in vivo in rats with vitamin D deficiency, as well as in **vitamin D receptor (Vdr)** knockout mice. Our data indicate that this is due to increased superoxide radical levels via down-regulation of secretory leukocyte protease inhibitor (SLPI), a secreted protein encoded by the *Slpi* gene (*SLPI* gene in humans). This protein protects epithelial tissues from several proteases and has anti-inflammatory and antioxidant properties (Gipson et al., 1999; Henriksen et al., 2004; Zhong et al., 2017). The expression of SLPI has also been reported to be regulated by vitamin D (Tarroni et al., 2012).

2 | METHODS

2.1 | Human tissues

Cavernosal specimens and blood samples were obtained from 12 organ donors at the time of organ collection for transplantation in the Hospital Universitario 12 de Octubre, Madrid, Spain. In addition to providing consent for organ transplantation, relatives provided informed consent for tissue procurement specifically for research purposes. The project protocol was approved by the ethics committees of the Hospital Universitario 12 de Octubre, Madrid, Spain (Ethics Approval Procedure 16/045), and Hospital Universitario Ramón y Cajal, Madrid, Spain (Ethics Approval Procedure 363-15). Subjects were only excluded if they had any infectious disease. Tissues were maintained in sterilized M-400 solution (composition per 100 ml: mannitol, 4.19 g; KH_2PO_4, 0.205 g; $K_2HPO_4{\cdot}H_2O$, 0.97 g; KCl, 0.112 g; and $NaHCO_3$, 0.084 g; pH 7.4, at 4–6°C). The time elapsed between harvesting of cavernosal specimens from organ donors to their functional evaluation ranged from 16 to 24 h. Within this time range, tissues remain totally viable and are adequate for functional evaluation (Angulo et al., 2019). Plasma was obtained from the 12 donors by centrifugation at 4°C of blood collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes.

2.2 | Animal model

Experimental procedures were approved by the Animal Welfare Body of Universidad Complutense de Madrid and Consejo Superior de Investigaciones Científicas and the regional committee of Comunidad de Madrid (Ref. PROEX-016/019 and PROEX-396.1/21) in accordance with the guidelines on the ethical use of animals from the European Community Council Directive of 22 September 2010 (2010/63/EU). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All investigators understand the ethical principles. All animals were kept under standard conditions of temperature 22 ± 1°C and 12:12-h dark/light cycle (standard white LED light conditions), with environmental enrichment, an individually ventilated cage (IVC), and free access to food and water. Rats were killed by decapitation and mice by cervical dislocation.

A pilot study was performed in CCs from wild-type (WT) male Sprague–Dawley rats with/without a vitamin D-free diet from a previous protocol (Olivencia et al., 2022). In addition, another batch of 2-month-old male Wistar rats were randomly allocated to two groups: rats fed with a standard diet (Teklad Global 18% Protein Rodent Diet, Envigo, n = 20) or a vitamin D-free diet (Teklad Custom Diet TD.120008, Envigo, n = 20) during 5 months. In vitro incubation experiments were performed with CCs obtained from control male Wistar rats (n = 14).

Vdr knockout mice were originally generated by Dr. Marie Demay (Harvard Medical School, Boston, MA, USA) (Li et al., 1997) and kindly donated to us to breed our own colony. The male *Vdr* knockout ($Vdr^{-/-}$, n = 14) and WT (n = 14) mice were obtained by crossing heterozygous mice ($Vdr^{+/-}$), genotyped as described (Protocol 22517 at http://www.jax.org) and used at 4 months of age. $Vdr^{-/-}$ mice were fed with a γ-irradiated diet (TD96348, Teklad, Madison, WI) containing 2% calcium, 1.25% phosphorus and 20% lactose to normalize the blood mineral ion levels (Li et al., 1998).

2.3 | Functional evaluation of CCs and penile resistance arteries

All functional experiments were performed in Krebs–Henseleit solution at 37°C, pH 7.4, and bubbled with 95% O_2 and 5% CO_2. Strips of human CC tissue (≈8 mm) from 11 patients were mounted in organ chambers and stretched to optimal isometric tension as described. An initial response to 80-mM KCl was recorded (59.8 ± 16.9 and 58.6 ± 203 mN, $P > 0.05$ low vs. high vitamin D). Neurogenic relaxations to electrical field stimulation (EFS, trains of 20-s duration at 180-s intervals with pulses of 0.5-ms duration and 75 mA at increasing rates from 0.5 to 16 Hz) were obtained in CC strips contracted with (5*Z*)-7-{(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxyoct-1-en-1-yl]-2-oxabicyclo [2.2.1]heptan-5-yl}hept-5-enoic acid (U46619) (10–30 nM, 41.8 ± 3.7 and 35.8 ± 11.2 mN, $P > 0.05$ low vs. high vitamin D). Penile small helicine arterial rings (lumen diameter 300–500 μm, 1.7–2.0 mm long) were dissected from seven CC specimens and mounted on microvascular wire myographs for isometric tension recordings as described (Olivencia et al., 2023). An initial response to 80-mM KCl was recorded (13.1 ± 14.2 and 19.7 ± 1.6 mN, $P > 0.05$ low vs. high vitamin D). The arteries were contracted with 1- to 3-μM **noradrenaline** (10.1 ± 4.2 and 12.7 ± 1.7 mN, $P > 0.05$ low vs. high vitamin D), and the relaxation was evaluated by cumulative addition of **acetylcholine** (ACh).

CC strips from rats (≈2 mm) and mice (≈1.5 mm) were suspended between two electrodes placed in a wire myograph, stretched to 3 mN. An initial response to 80-mM KCl was recorded (3.52 ± 0.6 and 3.49 ± 0.9 mN, $P > 0.05$ control vs. vitamin D-free diet rats). Then, CCs were incubated with **guanethidine** (10 μM) and **atropine** (1 μM) to block adrenergic neurotransmission and **muscarinic** receptors, respectively, and contracted with **phenylephrine** (0.3–1 μM, 3.50 ± 0.6 and 3.31 ± 0.4 mN, $P > 0.05$ control vs. vitamin D-free diet rats, respectively). Relaxations were induced by EFS stimulation (trains of 10-s duration at 180-s intervals with pulses of 1-ms duration and 20%

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above the threshold current intensity at increasing rates from 0.5 to 16 Hz) or by vasoactive agents (Olivencia et al., 2023). Rat dorsal penile arterial segments (2 mm long) were mounted in a wire myograph and stretched to give an equivalent transmural pressure of 100 mmHg. Arteries were contracted with phenylephrine (1–3 μM), and relaxations were induced by ACh or **sildenafil**. All drugs were incubated for 10 min except PEGylated superoxide dismutase (PEGSOD) for 30 min and SLPI for 24 h as described below. Relaxations were expressed as a percentage of the reduction in vasoconstrictor-induced contraction.

In some experiments, rat CC strips were incubated with/without 0.166-μg·ml^{-1} secretory leukocyte protease inhibitor (SLPI, MyBioSource, Vancouver, Canada, Cat# MBS144051) in Dulbecco's modified Eagle's medium supplemented with 1% antibiotic–antimycotic solution, pyruvate solution and non-essential amino acids (Merck, Darmstadt, Germany) at 37°C, 95% humidity and 5% CO_2 for 24 h before mounting the strips in the myograph.

2.4 | *Slpi* mRNA silencing

CC strips were transfected with specific siRNA against *Slpi* (*Slpi* Rat siRNA Oligo Duplex [Locus ID 84386], OriGene, Rockville, USA) and control scramble non-targeting siRNA using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. The complex of siRNA with a final concentration of 50 nM and Lipofectamine was mixed with Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific) supplemented with 1% antibiotic–antimycotic solution and transfected into rat strips. Twenty-four hours after transfection, strips were mounted in the myograph or immediately frozen at −80°C.

2.5 | Intracavernosal pressure (ICP) recording

Rats and mice were anaesthetized with **ketamine** (60 mg·kg^{-1}) and **diazepam** (4 mg·kg^{-1}). The right cavernous nerve was dissected, and ICP, that is, the main outcome measure, was recorded by insertion of a 25-gauge needle into the right crus. Electrical stimulation was applied by a platinum bipolar hook electrode connected to a stimulator, and frequency–response curves were performed by applying stimulation at increasing frequencies at 3-min intervals (Angulo et al., 2005; Olivencia et al., 2023). The left carotid artery was catheterized in rats for constant blood pressure measurement. After ICP evaluation, rats and mice were killed by anaesthetic overdose. The absence of a heartbeat for 10 min was used to confirm the death.

2.6 | Histology

Tissue samples from the penis were either fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) and embedded in paraffin or cryoprotected in 30% sucrose in PBS, immersed in optimal cutting temperature (OCT) compound, snap-frozen in liquid nitrogen and stored at −80°C. Paraffin transversal penis sections were stained with Masson's trichrome staining technique and examined by light microscopy. Quantification of blue staining was performed in a blinded manner with Image-Pro Plus 2D image analysis software.

For immunostaining, OCT-embedded penis sections were permeabilized in PBS with 0.3% Triton X-100 at room temperature for 10 min, followed by 1-h incubation with blocking buffer containing 10% normal goat serum (Life Technologies, 16210-072, Carlsbad, CA, USA) and 0.1% Tween 20 in PBS at room temperature. Then, slices were incubated with primary antibodies overnight at 4°C. The following duplex immunofluorescence staining were performed: (1) anti-SLPI (1:50, mouse monoclonal, Santa Cruz Biotechnology, Dallas, USA, Cat# sc-374575) followed by Alexa Fluor 488-conjugated goat anti-mouse (1:200, Thermo Fisher Scientific, MA, USA, Cat# A21042, RRID:AB_2535711) and anti-α-smooth muscle actin CY3 (1:200, mouse monoclonal, Sigma-Aldrich, Darmstadt, Germany, Cat# C6198, RRID:AB_476856) and (2) anti-VDR (1:50, mouse monoclonal, Santa Cruz Biotechnology, Dallas, USA, Cat# sc-13133, RRID:AB_628040) followed by Alexa Fluor 594-conjugated goat anti-mouse (1:200, Thermo Fisher Scientific, Cat# A11032, RRID:AB_2534091) and anti-α-smooth muscle actin Alexa Fluor 488 (1:200, Thermo Fisher Scientific, Cat# 53-9760-82, RRID:AB_2574461). Anti-α-smooth muscle actin was added after a series of PBS washes following the first staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1.24653, Merck, Darmstadt, Germany), and slides were mounted with SlowFade Antifade Mounting Medium (S36937, Thermo Fisher Scientific).

Images were captured using a Zeiss laser scanning confocal microscope (LSM 710, Unidad de Citometría de Flujo y Microscopía de Fluorescencia, Universidad Complutense de Madrid). For Z-stack images, 4–8 consecutive XY images were obtained by a Zeiss confocal microscope (LSM 710). The images were analysed in a blinded manner using the ImageJ software.

2.7 | Superoxide measurements by dihydroethidium (DHE) and lucigenin chemiluminescence

OCT-embedded penis sections of 6–8 μm from humans and rats were cut in a cryostat and incubated with or without 4-hydroxy-TEMPO (TEMPOL; Merck, Darmstadt, Germany) at 10 mM for 30 min. Afterwards, slices were exposed to 3 μM (rats) or 4 μM (human) DHE (Merck, Darmstadt, Germany) for 30 min at 37°C (Angulo et al., 2019). Nuclei were stained with 1 μM DAPI for 5 min. All images were captured in a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). DHE intensity was obtained through ImageJ software and normalized with DAPI intensity.

CC strips were dissected and then transferred to microtitre plate wells containing 5-μM bis-*N*-methylacridinium nitrate (Lucigenin; Merck, Darmstadt, Germany); some strips were stimulated with NADPH (100 μM; Merck), and TEMPOL (1 mM) was used as a negative control. Chemiluminescence was measured in a luminometer

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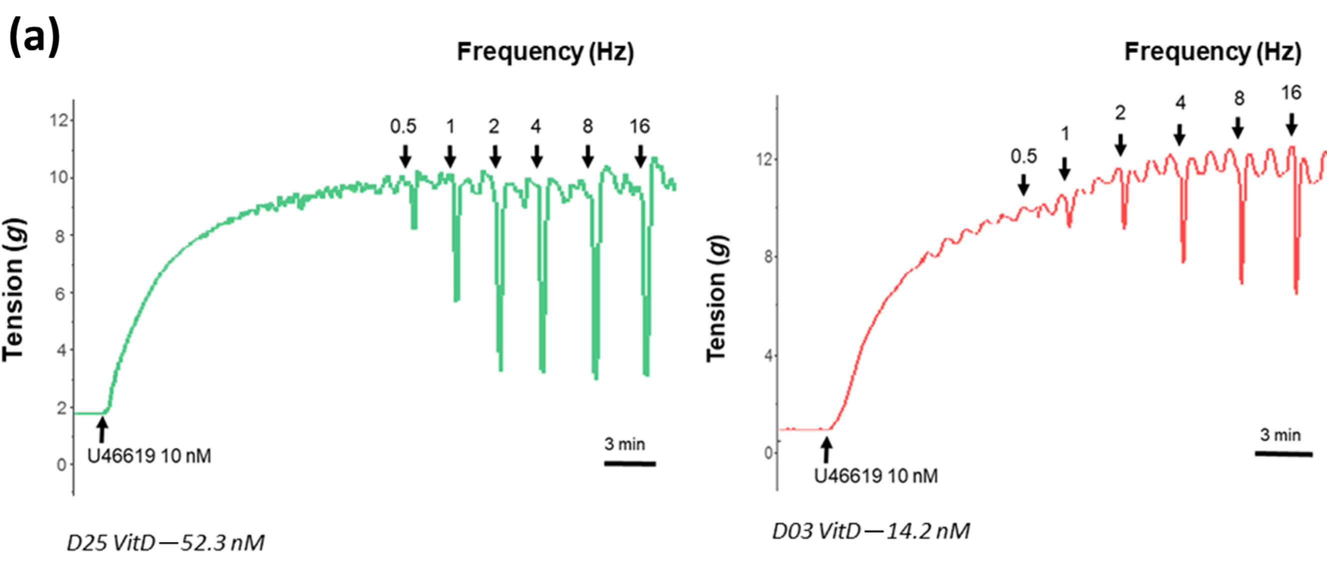
(BMG FLUOstar OPTIMA). Baseline values were subtracted from the counts obtained under the different experimental conditions, and superoxide production was normalized to dry tissue weight.

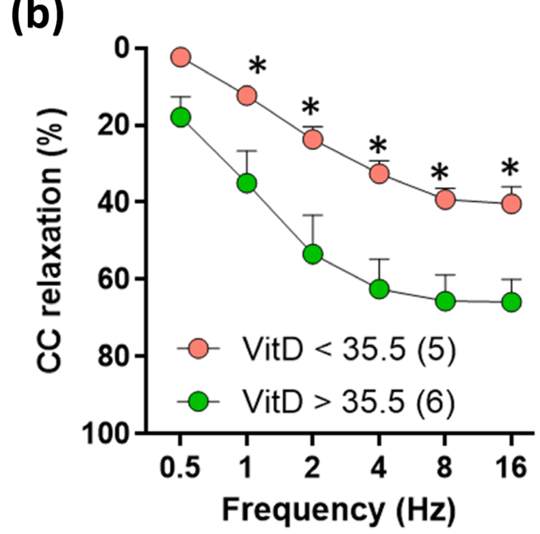
2.8 | Measurement of 25-hydroxyvitamin D, testosterone and SLPI in plasma

25-Hydroxyvitamin D, testosterone and SLPI levels were measured in rat citrated plasma and human EDTA plasma using a General 25-Hydroxyvitamin D3 (HVD3) ELISA Kit (Reddot Biotech Inc., Kelowna, Canada), Testosterone ELISA DE1559 (Demeditec, Kiel, Germany) and SLPI kit (DY1274-05, Bio-Techne R&D Systems, MN, USA) following the manufacturers' instructions.

2.9 | RNA sequencing (RNA-seq)

Library preparation and sequencing were carried out in Fundación Parque Científico de Madrid. Briefly, Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) was used for total RNA extraction following the manufacturers' recommendations (including





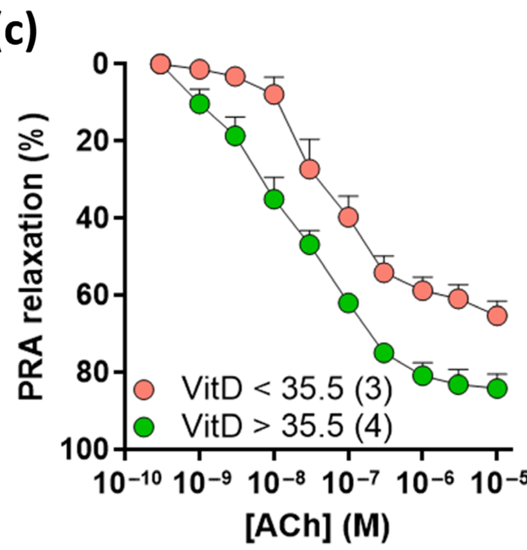


FIGURE 1 25-Hydroxyvitamin D plasma levels correlate with human corpus cavernosum (CC) and human penile resistance artery (PRA) function ex vivo. 25-Hydroxyvitamin D plasma levels were measured in donors, and CCs were divided into two groups: those from patients with 25-hydroxyvitamin D above, or those below, the median value (35.5 nM). (a) Original recordings of human CCs stimulated by electrical field stimulation (EFS) from a patient with normal (left) or low (right) 25-hydroxyvitamin D levels. (b) Relaxant responses of human CCs induced by EFS in the two groups. (c) Relaxant responses of human PRA induced by acetylcholine (ACh) in the two groups. Results are means ± standard error of the mean (SEM), and n is indicated in parentheses. *$P < 0.05$ versus low 25-hydroxyvitamin D by two-way (deficit × frequency or deficit × concentration) analysis of variance (ANOVA) test followed by Šidák's multiple comparisons test.

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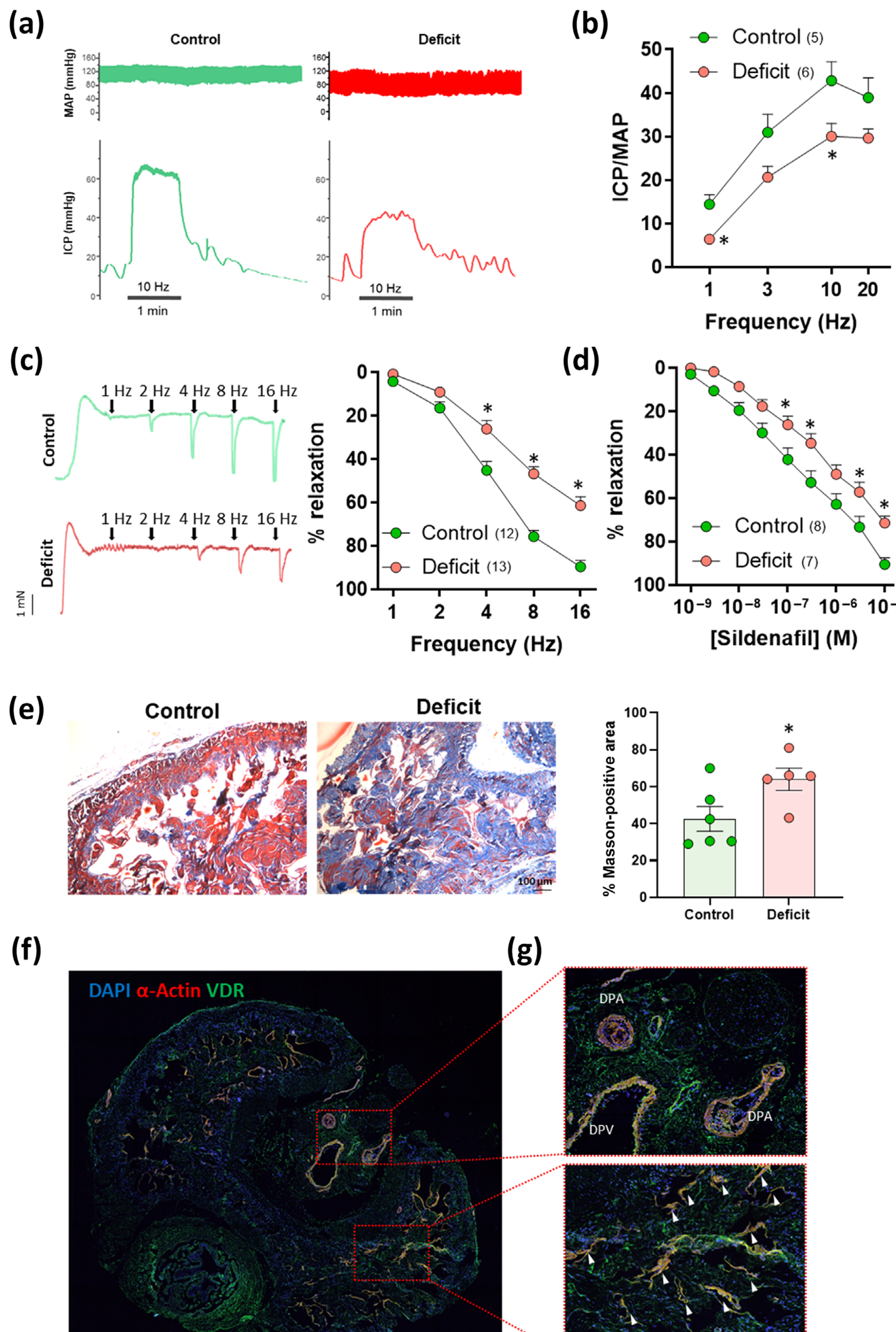

FIGURE 2 Legend on next page.

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DNase treatment). Once extracted, 100 pg of total RNA from each sample was used as input for library preparation with NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturers' recommendations. The so-obtained libraries were validated and quantified in a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and an equimolecular pool was made, purified using AMPure XP Beads (Beckman Coulter, Brea, CA, USA) and titrated by quantitative polymerase chain reaction (PCR) using the KAPA SYBR FAST qPCR Kit for LightCycler 480 and a reference standard for quantification. The library pool was denatured and loaded on a NovaSeq 6000 (Illumina, San Diego, CA, USA) where clusters were formed and sequenced using a single read format of 1 × 100 nt. The number of pass-filter clusters obtained per sample was 16–22 million, with an average of 19.3 million.

2.10 | RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from CC strips at the Unidad de Genómica (Parque Científico de Madrid). RNA quantity and quality were assessed with a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). One microgram of RNA was reverse-transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) following the manufacturers' instructions. *Slpi* gene expression was determined by qRT-PCR using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) with specific primers and a probe from Thermo Fisher Scientific databases (Rn07312880_g1). Amplifications, detections and analyses were performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The delta–delta Ct method was used to quantify relative changes in mRNA expression. Gene expression was normalized to the geometrical mean of *Actb* (Rn00667869_m1; Thermo Fisher Scientific, MA, USA) and *B2m* (Rn00560865_m1; Thermo Fisher Scientific) expression.

2.11 | Western blot

Penis tissues were homogenized with radioimmunoprecipitation assay (RIPA) (Merck, Darmstadt, Germany) and supplemented with protease (Protease Inhibitor Cocktail Tablets, Roche Diagnosis GmbH, Mannheim, Germany) and phosphatase inhibitor (PhosSTOP, Roche Diagnostics GmbH) cocktail in a glass potter homogenizer. Homogenates were run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes and incubated overnight with primary mouse antibodies against SLPI (1:500, Santa Cruz Biotechnology, Cat# sc-374575, RRID:AB_10989770) and vinculin (1:1000, Santa Cruz Biotechnology, Cat# sc-25336, RRID:AB_628438) and then with the secondary peroxidase-conjugated antibodies. Antibody binding was detected using an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Blots were imaged using an Odyssey Fc System (LI-COR Biosciences) and were quantified by densitometry using Quantity One software. Samples were normalized through expression of vinculin. The relative abundance of the protein of interest was normalized to the mean of the controls. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.12 | Statistics

Statistical analyses were performed using GraphPad Software v8 (GraphPad Software Inc., USA). All data were tested for normal distribution using the Shapiro–Wilk test, and parametric or non-parametric statistics were used as appropriate. ‘n’ indicates the number of animals. Data are presented either as scatter plots and means or as means ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) and, for multiple comparisons, the Šidák method or the Bonferroni post hoc test were used. Post-hoc tests were run only if F achieved $P<0.05$ and there was no significant variance inhomogeneity. *P* values of less than 0.05 were considered statistically significant. Pearson correlations were applied when relevant. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.13 | Nomenclature of targets and ligands.

Key protein targets and ligands in this paper are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos, et al., 2023; Alexander, Cidlowski, et al., 2023; Alexander, Fabbro, et al., 2023; Alexander, Kelly, et al., 2023).

FIGURE 2 Vitamin D deficiency induces erectile dysfunction (ED) in rats in vivo and ex vivo. (a) Original traces of the mean arterial pressure (MAP) and intracavernosal pressure (ICP) recordings after electrical stimulation of the cavernous nerve in anaesthetized rats at 10 Hz of frequency. (b) Averaged increases in ICP normalized to MAP. Effects of vitamin D deficiency on the relaxant responses of corpora cavernosa (CCs) induced by (c) electrical field stimulation (EFS) including original traces and (d) sildenafil. (e) Representative images of cross-sections of penises stained with Masson's trichrome. Extracellular collagen deposition was quantified as the percentage of blue over the total area; 4–7 images per animal were used. Results are means ± SEM, and n is indicated in parentheses. (f) Vitamin D receptor (VDR) localization by immunofluorescence labelling and confocal imaging in penis rat slices. (g) Amplification of sections from panel (f). VDR expression is shown in green, α-actin in red and nuclei in blue (4′,6-diamidino-2-phenylindole [DAPI]). DPA, dorsal penile artery; DPV, dorsal penile vein. Arrows indicate colocalization of VDR and α-actin. *$P < 0.05$ versus control using Student's *t* test (e) or otherwise using two-way (deficit × frequency or deficit × concentration) analysis of variance (ANOVA) test followed by Šidák's multiple comparisons test.

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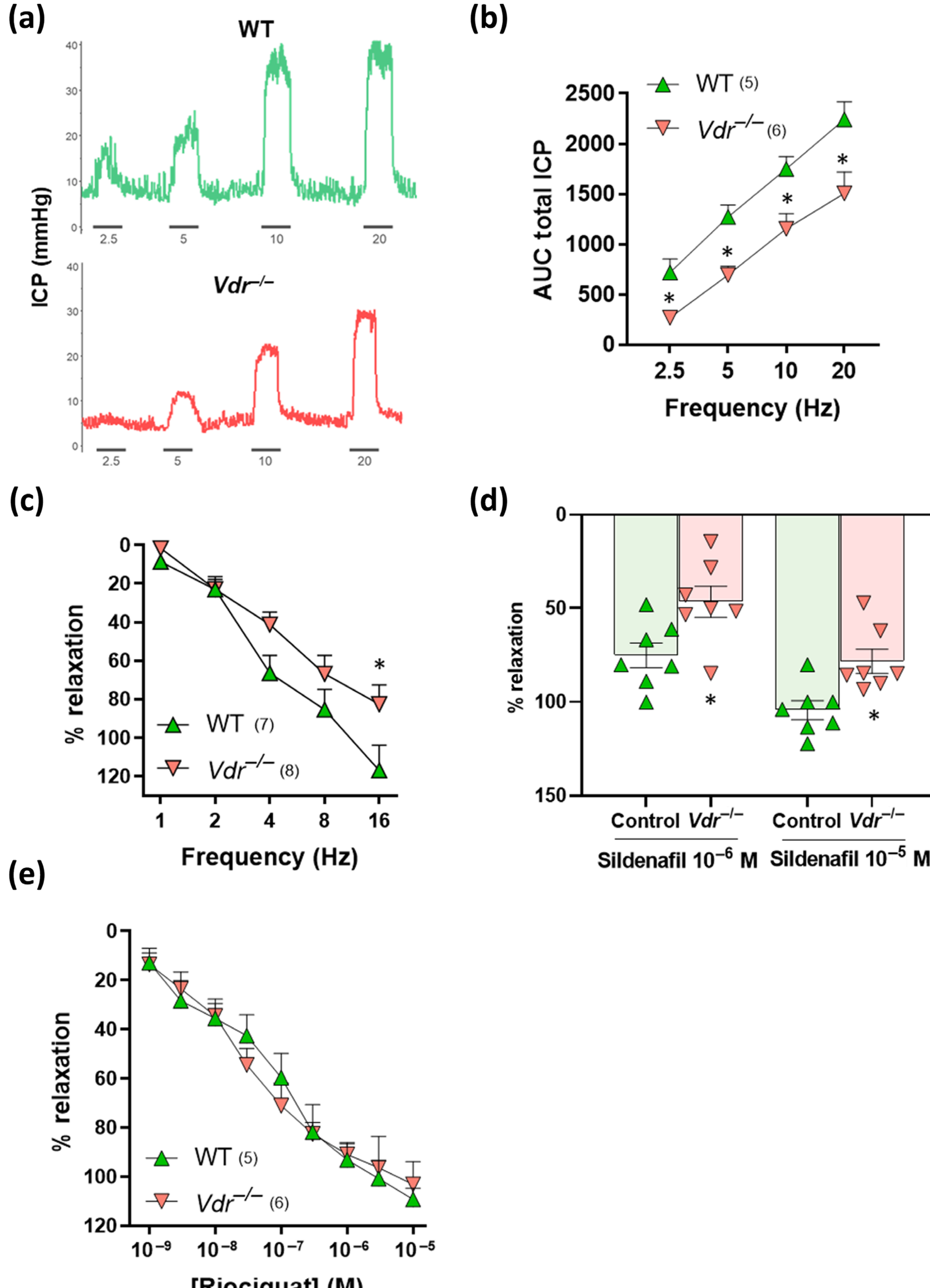

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3 | RESULTS

3.1 | Vitamin D deficiency is associated with erectile dysfunction (ED) in vitro in human donors

The aetiologies of ED include vascular, hormonal, neurological and/or psychological dysfunctions, which are often interlinked (Shamloul & Ghanem, 2013). We investigated whether the relationship between ED and vitamin D deficiency, as reported in epidemiological studies, could also be observed ex vivo, that is, in the absence of hormonal or psychological influences. Therefore, we analysed 25-hydroxyvitamin D levels in the plasma from organ donors and compared them with the relaxant responses induced by EFS in their isolated human CCs. Donor characteristics are shown in Table S1 (Angulo et al., 2019). EFS induces the release of NO from nitrergic nerves, leading to arterial vasodilation and relaxation of the trabecular smooth muscle cells of the CCs. This process increases ICP and causes penile engorgement. Endothelium- and NO-dependent vasodilation of penile arteries is also an important contributor to penile erection. Consequently, EFS-induced relaxation serves as an ex vivo surrogate test for erectile function, while ACh-induced vasodilation represents a standard test to analyse endothelial function. In agreement with the high prevalence of both ED and vitamin D deficiency, many donors exhibited moderately or severely reduced 25-hydroxyvitamin D levels and/or reduced responses to EFS in CCs (Figure 1). Both parameters were normally distributed in the cohort. Erectile function was markedly different in patients with 25-hydroxyvitamin D levels above versus below the median value (35.5 nM) of the cohort (Figure 1a,b). The median 25-hydroxyvitamin D levels also seem to discriminate between those with high and low endothelial-dependent response (Figure 1c). However, testosterone in plasma did not correlate with 25-hydroxyvitamin D levels (Figure S1A). Thus, the results suggest that the association of vitamin D deficiency with poor erectile function remains in isolated human CCs. We must emphasize that due to this small sample size, the lack of correlation with testosterone should be interpreted with caution.

3.2 | Vitamin D deficiency induces in vivo and ex vivo erectile dysfunction (ED) and penile fibrosis

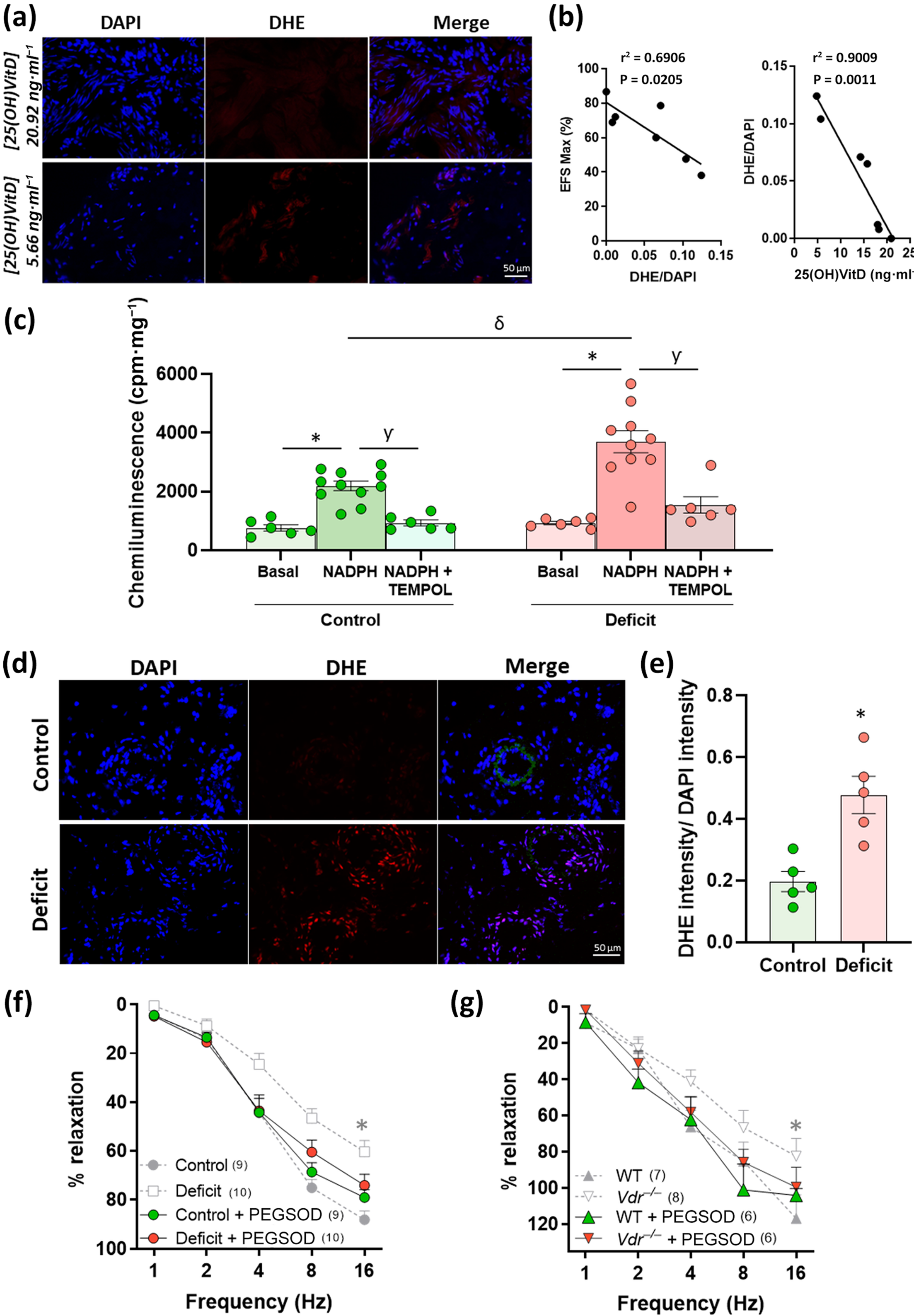
To analyse the cause–effect relationship, in parallel with the pulmonary study of a previous project (Olivencia et al., 2022), we evaluated the erectile function in wild-type Sprague rats exposed to a vitamin D-free diet for 5 months, compared with a standard diet. These preliminary results indicate that a decrease in 25-hydroxyvitamin D levels (24.0 ± 1.2 nM in vitamin D-free vs. 63.65 ± 2.3 nM in standard diet-fed rats [mean ± SEM]) without changes in plasma calcium (1.4 ± 0.01 vs. 1.4 ± 0.01 mM, respectively) (Olivencia et al., 2022) results in a significant reduction in EFS-induced relaxation (Figure S2A). In addition to neuronal-derived NO, NO released from the endothelium of sinusoids and blood vessels may also be involved in penile erection. Vitamin D deficiency also induces a significant decrease in the endothelium- and NO-dependent relaxation induced by ACh (Figure S2B). Moreover, the relaxation induced by the PDE5i sildenafil, which potentiates endogenous NO, is also reduced (Figure S2C). However, the relaxations to the soluble guanylyl cyclase (sGC) stimulator riociguat are unchanged (Figure S2D). The relaxation induced by EFS is almost entirely inhibited by the NO synthesis inhibitor L-nitro-Arg and potentiated by sildenafil in both groups (Figure S3). In addition, the relaxation to ACh (Figure S4A) and sildenafil (Figure S4B) in the dorsal penile arteries is also reduced in animals with vitamin D deficiency.

We designed a new set of experiments with Wistar rats exposed for 5 months to a vitamin D-free diet to analyse the erectile function in vivo and to gain insights into the mechanisms involved in ED. This intervention significantly reduces the 25-hydroxyvitamin D plasma levels but does not affect those of testosterone (Figure S5). Notably, the increase in ICP induced by electrical stimulation of the cavernosal nerve in anaesthetized rats is markedly decreased by vitamin D deficiency (Figure 2a,b). In addition, CC strips isolated from vitamin D-deficient rats also show reduced relaxation induced by EFS (Figure 2c) and by the PDE5i sildenafil (Figure 2d). Therefore, vitamin D deficiency induces ED ex vivo, confirming the pilot study, and in vivo, it was associated with reduced response to sildenafil, the first-choice therapeutic drug for ED.

Penile cavernous fibrosis is considered an important factor leading to ED. Therefore, we analysed the collagen deposition in paraffin penis sections by Masson's trichrome staining as a marker of fibrosis. We find that vitamin D deficiency significantly increases the blue-stained area, indicating excess deposition of collagen in the CCs (Figure 2e).

The active form of vitamin D, calcitriol, activates the VDR, a member of the nuclear receptor superfamily of transcription factors that regulate gene expression. To confirm that the VDR is present in the rat penis, its expression was analysed in sections of penises from Wistar rats by immunohistochemistry (IHC). Figure 2f shows a positive staining for the VDR in cavernosal smooth muscle cells, the dorsal penile arteries and the dorsal penile vein where it colocalizes with smooth muscle α-actin.

FIGURE 3 Vitamin D receptor (VDR) knockout induces erectile dysfunction (ED) in mice ex vivo and in vivo. (a) Original traces of intracavernosal pressure recording after electrical stimulation of the cavernous nerve in anaesthetized wild-type (WT) and *Vdr*$^{-/-}$ mice with increasing frequencies. (b) Averaged increases in pressure. Effects of *Vdr* deletion on the relaxant responses of corpora cavernosa (CCs) induced by (c) electrical field stimulation (EFS), (d) sildenafil and (e) riociguat. A full frequency or concentration–response curve was performed for EFS and riociguat, but only two concentrations of sildenafil were tested because the responses were slower to this drug. Results are means ± SEM, and n is indicated in parentheses. *$P < 0.05$ versus WT using two-way (deficit × frequency or deficit × concentration) analysis of variance (ANOVA) test followed by Šidák's multiple comparisons test. AUC, area under the curve; ICP, intracavernosal pressure.



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3.3 | *Vdr* knockout mice exhibit erectile dysfunction (ED) ex vivo and in vivo

Then, we analyse whether the ED induced by vitamin D deficiency could also be mimicked by genetic deletion of its receptor. In vivo and ex vivo erectile function was explored in *Vdr* knockout ($Vdr^{-/-}$) and WT mice. As shown in the original recordings (Figure 3a) and in the averaged data (Figure 3b), the increase in ICP in response to electrical stimulation is lower at all electrical frequencies in $Vdr^{-/-}$ mice compared to WT, confirming the ED in vivo. Furthermore, CCs isolated from $Vdr^{-/-}$ mice show decreased relaxation induced by EFS (Figure 3c) and by the PDE5i sildenafil (Figure 3d), also corroborating the ED ex vivo. However, as in vitamin D-deficient rats, there are no changes in the response to the sGC stimulator riociguat in $Vdr^{-/-}$ mice (Figure 3e).

3.4 | Excess superoxide mediates vitamin D deficiency- and *Vdr* deletion-induced erectile dysfunction (ED)

The reduced responses to EFS and sildenafil in vitamin D-deficient rats and $Vdr^{-/-}$ mice, which are dependent on endogenous NO, and the unaffected response to riociguat, which mimics NO but does not require endogenous NO, gave us a clue of a possible mechanism: a reduced bioavailability of NO due to excess levels of superoxide anion, which is a well-known mechanism involved in ED (Jeremy et al., 2007). Thus, we measured superoxide production in human penile sections by DHE fluorescence and compared these data with the ex vivo function of the CCs and the 25-hydroxyvitamin D levels in each donor. As expected, donor tissue sections with higher intensity of DHE fluorescence exhibit reduced maximal relaxant response to EFS (Figure 4a); that is, the higher the superoxide, the worse the erectile function. Interestingly, the DHE signal also correlates with the levels of 25-hydroxyvitamin D (Figure 4b).

To explore the causal relationship between 25-hydroxyvitamin D and superoxide, we measured superoxide levels by both lucigenin luminescence and DHE staining in CCs isolated from control and vitamin D-deficient rats. In CC strips from vitamin D-deficient rats, the basal lucigenin signal is not significantly different from the control strips (Figure 4c), but the NADPH-induced increase is significantly higher in CCs from vitamin D-deficient rats. In both experimental groups, the antioxidant TEMPOL prevents the NADPH-induced signal, indicating that it is specific for superoxide production. Similarly, the penises from vitamin D-deficient rats show marked red DHE nuclear staining (Figure 4d,e), again indicating high superoxide levels compared to controls.

To explore whether increased superoxide generation is involved in the ED, we analysed the response to EFS of CC strips from control and vitamin D-deficient rats and from $Vdr^{-/-}$ and WT mice incubated with PEGSOD. This antioxidant significantly reverted the in vitro ED induced by both vitamin D deficiency in rats (e.g., 31.5 ± 5.2% vs. 6.0 ± 5.7% inhibition at 16 Hz in the absence and presence of PEGSOD, respectively, Figure 4f) and genetic ablation of the VDR receptor in mice (e.g., 29.5 ± 8.5% vs. 4.4 ± 10% inhibition, respectively, Figure 4g). Therefore, these data strongly suggest that vitamin D deficiency- and VDR ablation-induced ED is mediated by increased superoxide production.

3.5 | Transcriptomic analysis of rat corpora cavernosa (CCs) with vitamin D deficiency

The VDR is a nuclear receptor that interacts with multiple vitamin D response elements (VDREs) present in the promoter region of its target genes, inducing or repressing their expression in a tissue-specific manner. To search for potential target genes and proteins involved in the mechanism of vitamin D deficiency-induced ED, we compared the transcriptome of CCs from control and vitamin D-deficient Wistar rats analysed by RNA-seq. Although principal component analysis (PCA) showed no significant differences between the vitamin D-deficient and control CCs (Figure 5a), several genes were significantly down-regulated or up-regulated by vitamin D deficiency (Figure 5b). Among these, we focused on *Slpi* because it encodes for the protein antileukoproteinase, also called secretory leukocyte protease inhibitor (SLPI), an inhibitor of serine proteases with antioxidant properties.

We confirm that *Slpi* was down-regulated in the CCs from the vitamin D-deficient rats at the level of mRNA by real-time PCR (RT-PCR) (Figure 5c) and protein by Western blot (Figure 5d). We also found that SLPI is expressed in the smooth muscle cells of the rat CCs (Figure 5e) by immunohistochemistry (IHC). To analyse whether altered SLPI could also be present in humans with vitamin D

FIGURE 4 Vitamin D deficiency- and vitamin D receptor gene (*Vdr)* deletion-induced erectile dysfunction is mediated by increased superoxide. (a) Dihydroethidium (DHE) staining in the corpora cavernosa (CCs) of a donor with high (above) and low (below) 25-hydroxyvitamin D. (b) Correlation between DHE intensity normalized by 4′,6-diamidino-2-phenylindole (DAPI) (DHE/DAPI) and the maximal effect of electrical field stimulation (EFS Max) (left) and between 25-hydroxyvitamin D plasma levels and DHE/DAPI (right) in CCs of human donors. (c) Levels of superoxide ($O_2^{\bullet -}$) measured by lucigenin chemiluminescence in rat CC strips in the absence (basal) or in the presence of NADPH or NADPH plus 4-hydroxy-TEMPO (TEMPOL). (d) Rat penis slices showing the blue fluorescence of the nuclear stain DAPI (left), the red fluorescence produced when DHE is oxidized to ethidium by superoxide (middle) and the merged images (right). (e) Values of DHE/DAPI; 4–10 images were taken from each animal. Effects of the relaxant responses of CCs induced by EFS in the presence or absence of the antioxidant PEGylated superoxide dismutase (PEGSOD) from (f) vitamin D-deficient rats or (g) *Vdr* knockout mice. The results from Figures 2c and 3c are superimposed in light grey and discontinuous lines just for reference. Results are means ± SEM, and n is indicated in parentheses. $^{*}P < 0.05$ versus basal or control; $^{\Upsilon}P < 0.05$ versus NADPH; $^{\delta}P < 0.05$ versus control NADPH using one-way analysis of variance (ANOVA) test followed by a Bonferroni post hoc test, except for Student's t test in (c).

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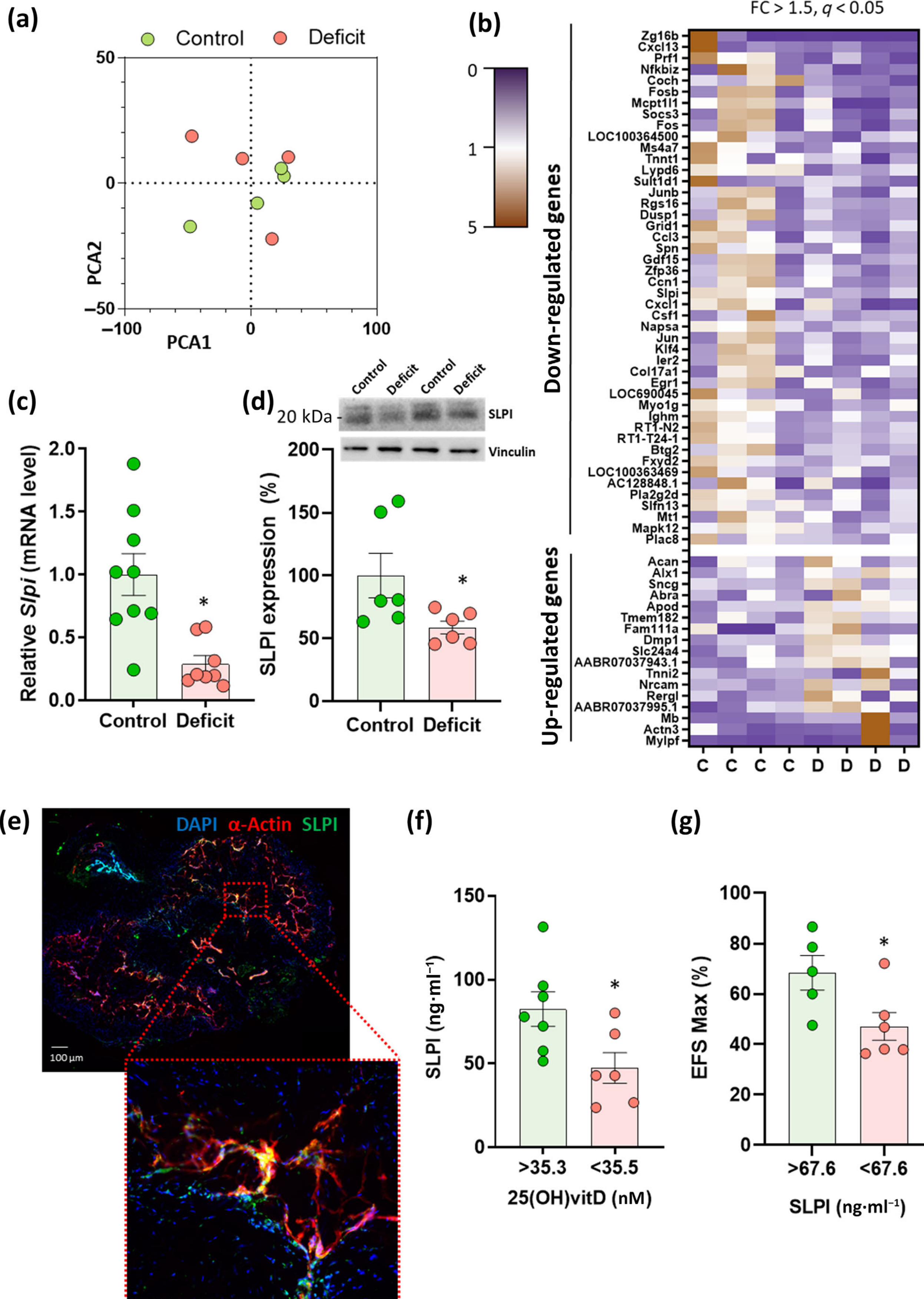

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deficiency and/or reduced erectile function, we measured SLPI levels in plasma from the human donors. Remarkably, donors with reduced vitamin D also show reduced SLPI (Figure 5f). Moreover, CCs from donors with SLPI levels below the median of the cohort (67.6 ng·ml^{-1}) show reduced relaxant response to EFS (Figure 5g).

3.6 | SLPI rescues superoxide-induced erectile dysfunction (ED), and *Slpi* gene knockdown induces ED in a superoxide-dependent manner

To assess whether SLPI can modulate erectile function and its relationship with superoxide, we incubated healthy rat CC strips with recombinant SLPI or vehicle for 24 h. These CCs were then exposed to vehicle or the superoxide generator pyrogallol in the presence or absence of PEGSOD (Figure 6a,b, with the calculated area under the curve (AUC) for both panels shown in Figure 6c). When CCs are incubated for ≥24 h, the relaxant response to EFS is almost absent due to neural degeneration in culture, and therefore, in these experiments, we only analysed the concentration–response curves to the NO donor diethylamine NONOate (DEA-NO). As expected, pyrogallol reduced the response to DEA-NO due to the rapid reaction of NO with superoxide. Interestingly, the pre-incubation with SLPI does not modify the response to DEA-NO but partially reverts the pyrogallol-induced ED. Furthermore, PEGSOD also prevents the in vitro ED induced by pyrogallol, and SLPI does not further increase DEA-NO relaxation in the presence of PEGSOD plus pyrogallol. Thus, exogenous SLPI mimics the effects of PEGSOD.

To investigate the role of endogenous SLPI in erectile function, we silenced *Slpi* in CC strips from healthy rats using a specific siRNA. A scramble siRNA served as a negative control. We achieved a >60% *Slpi* mRNA down-regulation (Figure 6d, inset), that is, yielding levels similar to those found in vitamin D-deficient rats. *Slpi* silencing results in a significant decrease in the relaxant response to DEA-NO (Figure 6d), indicating that SLPI is necessary for proper erectile function. Moreover, the superoxide scavenger PEGSOD reverts the inhibitory effect of *Slpi* siRNA (Figure 6e,f), indicating that superoxide is involved in this effect.

4 | DISCUSSION

Previous epidemiological studies have linked vitamin D status with erectile function (Canguven & Al Malki, 2021; Demirci et al., 2021; Horsanali et al., 2020). Here, we report for the first time a direct correlation between vitamin D levels and EFS-induced relaxation in isolated CCs as well as endothelial function in isolated resistance penile arteries from human donors. Additionally, we demonstrate, in rats and mice, a causal relationship between exposure to a vitamin D-free diet or *Vdr* knockout and ED both ex vivo and in vivo. Notably, our findings suggest that increased superoxide is involved in vitamin D deficiency-mediated ED and that down-regulation of SLPI is a novel underlying mechanism to induce ED.

Vitamin D deficiency may potentially impact several factors that are known to be involved in ED. Thus, low levels of 25-hydroxyvitamin D have been reported to be linked to depressive disorders (May et al., 2010) and to low testosterone levels (Wehr et al., 2010), which are known causes for psychogenic and hormonal ED, respectively. Our results indicate that the cause of the failure in penile erection may be intrinsic to the CCs and that neither human nor rat testosterone levels are influenced by 25-hydroxyvitamin D levels. Consequently, vitamin D deficiency is associated with ED of vascular origin, although psychogenic and hormonal aetiologies cannot be completely ruled out. Nevertheless, our studies in human CCs must be interpreted with caution because of the relatively small sample size and the observational nature of the findings, which preclude establishing causation. However, rat models exposed to vitamin D-free diets exhibited 25-hydroxyvitamin D levels comparable to the lower range observed in human subjects with ED, making this model suitable for further exploration of the condition. Furthermore, recent studies show that vitamin D_3 supplementation above standard levels enhances erectile function in a rat model of cavernous nerve injury, underscoring the therapeutic potential of vitamin D (Zhu et al., 2023).

The active form of vitamin D, calcitriol, binds to the VDR, which functions as a transcription factor and interacts with the VDREs in the promoter regions or its target genes to regulate gene expression (Carlberg & Campbell, 2013; Dusso et al., 2005). In this study, we observed ubiquitous VDR expression in the penis and, in particular, in the smooth muscle cells of the vessels and the CCs. Although we did not colocalize the VDR with specific endothelial markers, the available single-cell RNA-seq (sc-RNAseq) data indicate that the VDR expression in endothelial cells is negligible in the human CCs (Zhao et al., 2022). This observation aligns with previous findings of low VDR expression in pulmonary artery endothelial cells compared to smooth muscle cells (Callejo et al., 2024). Interestingly, our results indicate that the absence of the VDR causes ED in vivo and ex vivo.

FIGURE 5 Transcriptomic changes in corpora cavernosa (CCs) from vitamin D-deficient rats. (a) Principal component analysis (PCA). (b) Heat map of significant down-regulated and up-regulated genes. FC, fold change. (c) *Slpi* mRNA expression in rat CCs by quantitative real-time polymerase chain reaction (qRT-PCR). (d) Averaged densitometric protein expression of secretory leukocyte protease inhibitor (SLPI) in homogenized penis analysed by Western blot and normalized to vinculin expression. (e) SLPI localization by immunofluorescence labelling and confocal imaging in rat penis slices. SLPI expression is shown in green, α-actin in red and nuclei in blue (4′,6-diamidino-2-phenylindole [DAPI]). (f) SLPI levels in plasma from human donors with 25-hydroxyvitamin D levels below and above the median of the cohort (35.5 nM). (g) Maximal response to electrical field stimulation (EFS) in human donor CCs with SLPI levels below and above the median of the cohort (67.6 ng·ml^{-1}). Results are means ± SEM. *P < 0.05 versus control by Student's t test.

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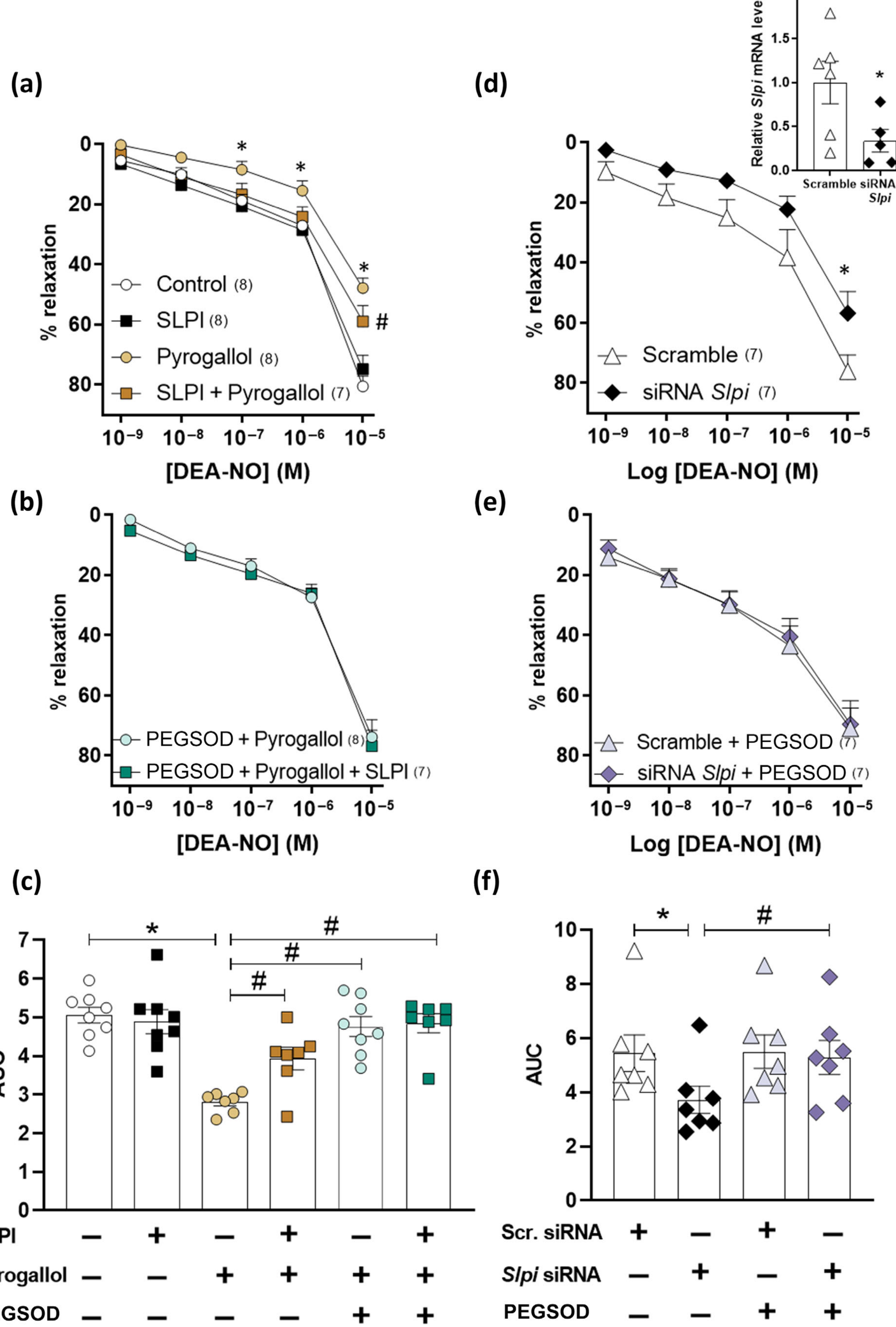

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Penile cavernous fibrosis is considered an important factor leading to ED. The tumour growth factor-β (TGF-β) and its downstream signalling partners p-SMAD2 and p-SMAD3 play pathophysiological roles in neurogenic (Canguven et al., 2009) and vasculogenic (Zhang et al., 2008) models of ED. Activated TGF-β signalling has also been observed in smooth muscle and endothelial cells from patients with ED (Zhao et al., 2022). Likewise, we also found fibrosis with increased collagen deposition in the CCs from vitamin D-deficient rats. This is consistent with the well-known anti-fibrotic effect of vitamin D in other organs such as the liver (Abramovitch et al., 2011), lung (Han et al., 2021) and kidney (Zhang et al., 2021), which is widely attributed to its double, VDR-dependent and VDR-independent, inhibitory effect on TGF-β signalling (Ding et al., 2013).

The impairment of the NO pathway is a recognized pathogenetic factor in ED in both animal models and human patients (Angulo et al., 2005, 2010; Gratzke et al., 2010). As expected, our findings confirm that the relaxation induced by EFS in both experimental groups is NO-dependent, as it is supressed by the NO synthesis inhibitor L-nitro-Arg. Likewise, sildenafil, which inhibits cGMP degradation, the downstream effector of NO, potentiates EFS responses in both groups. Moreover, vitamin D deficiency significantly reduces the responses to ACh and sildenafil in both the CCs and the dorsal penile artery. Accordingly, the relaxation of the sGC stimulator riociguat in CCs, which does not rely on endogenous NO, was unaffected by vitamin D deficiency or *Vdr* ablation. These results are consistent with a reduced bioavailability of NO underlying ED induced by vitamin D deficiency.

Our finding that vitamin D deficiency reduces the response to PDE5i in both the CCs and the penile arteries may have important clinical implications. For unclear reasons, many patients with ED are non-responders to PDE5i (Park et al., 2013). Our results suggest that vitamin D deficiency may contribute to the lack of response to this first-line treatment of ED. Likewise, addition of vitamin D to the PDE5i tadalafil increased the erectile function in patients with ED (Demirci et al., 2021). Similarly, vitamin D deficiency reduces the effect of sildenafil in rat pulmonary arteries and is a good predictor of poor therapeutic response to PDE5i in patients with pulmonary arterial hypertension (Callejo et al., 2021).

Oxidative stress, primarily driven by superoxide production, is a critical factor in the development of ED. NADPH oxidase has been identified as a major source of superoxide in the CCs in vascular ED (Jeremy et al., 2007; Li et al., 2012, 2015). Accordingly, our results of increased NADPH-stimulated lucigenin luminescence and DHE fluorescence indicate an increase in superoxide penile production after chronic vitamin D deficiency in both rats and humans. The functional experiments with PEGSOD confirm the causative role of superoxide in vitamin D deficiency- or *Vdr* knockout-induced ED.

We wondered which potential pathways are involved in the redox imbalance caused by vitamin D deficiency in the penis. A large number of genes are potentially regulated by vitamin D, and these are tissue-specific (Wang et al., 2005). We performed a transcriptomic analysis in CCs from control and vitamin D deficiency animals. We found 46 down-regulated and 17 up-regulated genes in vitamin D-deficient rat CCs. Notably, there was an up-regulation of actin-binding rho-activating protein (*Abra*) gene that has been related with smooth muscle cell proliferation (Troidl et al., 2009) and aortic endothelial dysfunction and whose expression has been reported to be controlled by vitamin D in the rat aorta (Wu-Wong et al., 2015). Myoglobin gene (*Mb*) expression was also up-regulated. Free myoglobin and haemoglobin are well-known quenchers of NO and thus a possible cause for ED (Murad et al., 1978).

Nevertheless, we focused on the down-regulation of the *Slpi* gene for further study due to the following reasons. First, SLPI reduces reactive oxygen species production (Kongpol et al., 2019; Nernpermpisooth et al., 2017) and increases superoxide dismutase 2 (SOD-2) expression (Kozin et al., 2017). SLPI expression is positively regulated by the antioxidant system **NRF2** (Harada et al., 2012; Schafer et al., 2012), which, in turn, is a target of the VDR (Montenegro et al., 2019) and can restore erectile function in rats and humans (Angulo et al., 2019). Second, SLPI also has multiple roles in inflammation, the resolution of inflammation, atherosclerosis and fibrosis (Gipson et al., 1999; Henriksen et al., 2004; Zhong et al., 2017). Interestingly, SLPI is down-regulated specifically in the smooth muscle cells of CCs from diabetic patients with ED (Zhao et al., 2022). Therefore, we first found that SLPI is expressed specifically in cavernosal smooth muscle cells by immunofluorescence and confirmed that it is down-regulated in vitamin D-deficient CCs by both RT-PCR and Western blot. We demonstrated the functional role of SLPI by (1) the protective effect of recombinant SLPI on exogenous superoxide-induced ED and (2) the superoxide-dependent deleterious effect of *Slpi* silencing on erectile function.

The expression of SLPI has also been reported to be regulated by calcitriol in human osteoblasts (Tarroni et al., 2012). However, we could not find a consensus VDRE in the *SLPI* gene promoter region, suggesting that SLPI expression is not directly regulated by the VDR

FIGURE 6 Recombinant secretory leukocyte protease inhibitor (SLPI) rescues superoxide-induced erectile dysfunction (ED), and *Slpi* gene knockdown induces ED in a superoxide-dependent manner. (a–c) Effects of the superoxide generator pyrogallol on the relaxant corpus cavernosum (CC) responses induced by the nitric oxide (NO) donor diethylamine NONOate (DEA-NO) in CCs from healthy animals incubated in the presence or absence of recombinant SLPI (0.166 μg·ml^{-1}, for 24 h), in the presence or absence of pyrogallol (0.1 mM), and in the absence (a) or presence (b) of PEGylated superoxide dismutase (PEGSOD) (30 U·ml^{-1}). (c) The area under the curve (AUC) calculated from the data in (a) and (b). (d–f) Effects of *Slpi* knockdown using *Slpi* siRNA or scramble siRNA in the absence (d) or presence (e) of PEGSOD. The real-time polymerase chain reaction (RT-PCR) verification of *Slpi* silencing is shown as an inset in (d). (f) The AUC calculated from the data in (d) and (e). Results are means ± SEM, and n is indicated in parentheses. $^{*}P < 0.05$ versus control or scramble and $^{\#}P < 0.05$ versus control or scramble using Student's *t* test (for mRNA) or two-way (treatment × concentration) analysis of variance (ANOVA) test followed by Šidák's multiple comparisons test. AUCs were compared by two- or three-way ANOVA followed by a Bonferroni test.

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but rather via other factors that, in turn, regulate SLPI expression. A decrease in the VDR target gene *NRF2*, which positively regulates SLPI expression (Harada et al., 2012; Schafer et al., 2012), and/or an increase in TGF-β, which is inhibited by the VDR and negatively regulates SLPI (Jaumann et al., 2000), might account, among other factors, for the down-regulated SLPI expression observed in vitamin D-deficient rats.

In conclusion, we demonstrate for the first time that vitamin D deficiency or *Vdr* knockout induces ED via increased superoxide and down-regulation of SLPI. We further suggest that vitamin D deficiency is an aetiological factor for vascular ED and for the therapeutic failure of PDE5i. Moreover, our results raise the possibility that restoring the vitamin D status in patients with vitamin D deficiency and ED would improve not only its calcium metabolism and bone health but also its sexual performance and/or the efficacy of the treatment for ED.

AUTHOR CONTRIBUTIONS

M. A. Olivencia: Formal analysis (equal); investigation (equal); writing—original draft (equal). **B. Climent:** Formal analysis (equal); investigation (equal). **B. Barreira:** Formal analysis (equal); investigation (equal). **D. Morales-Cano:** Formal analysis (equal); investigation (equal). **A. Sánchez:** Investigation (equal). **A. Fernández:** Investigation (equal). **B. García-Gómez:** Resources (equal). **J. Romero-Otero:** Resources (equal). **C. Rodríguez:** Investigation (equal). **L. Moreno:** Supervision (equal); writing—review and editing (equal). **D. Prieto:** Supervision (equal). **M. J. Larriba:** Investigation (equal); resources (equal). **A. Cogolludo:** Supervision (equal); writing—review and editing (equal). **J. Angulo:** Conceptualization (equal); supervision (equal); writing—review and editing (equal). **F. Perez-Vizcaino:** Conceptualization (equal); funding acquisition (equal); supervision (equal); validation (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

MAO and FP-V have applied for an international patent for the therapeutic use of SLPI. All other authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

Data are available from the authors on reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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REFERENCES

Abramovitch, S., Dahan-Bachar, L., Sharvit, E., Weisman, Y., Ben Tov, A., Brazowski, E., & Reif, S. (2011). Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. *Gut*, *60*(12), 1728–1737. https://doi.org/10.1136/gut.2010.234666

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Abbracchio, M. P., Abraham, G., Agoulnik, A., Alexander, W., Al-Hosaini, K., Bäck, M., Baker, J. G., Barnes, N. M., … Ye, R. D. (2023). The Concise Guide to PHARMACOLOGY 2023/24: G protein-coupled receptors. *British journal of pharmacology*, *180*, S23–S144. https://doi.org/10.1111/bph.16177

Alexander, S. P. H., Cidlowski, J. A., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Coons, L., Fuller, P. J., Korach, K. S., & Young, M. J. (2023). The Concise Guide to PHARMACOLOGY 2023/24: Nuclear hormone receptors. *British Journal of Pharmacology*, *180*(S2), S223–S240. https://doi.org/10.1111/bph.16179

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Annett, S., Boison, D., Burns, K. E., Dessauer, C., Gertsch, J., Helsby, N. A., Izzo, A. A., Ostrom, R., Papapetropoulos, A., … Wong, S. S. (2023). The concise guide to PHARMACOLOGY 2023/24: Enzymes. *British Journal of Pharmacology*, *180*, S289–S373. https://doi.org/10.1111/bph.16181

Alexander, S. P. H., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Buneman, O. P., Faccenda, E., Harding, S. D., Spedding, M., Cidlowski, J. A., Fabbro, D., Davenport, A. P., Striessnig, J., Davies, J. A., Ahlers-Dannen, K. E., Alqinyah, M., Arumugam, T. V., Bodle, C., … Zolghadri, Y. (2023). The Concise Guide to PHARMACOLOGY 2023/24: Introduction and Other Protein Targets. *British Journal of Pharmacology*, *180*, S1–S22. https://doi.org/10.1111/bph.16176

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the *British Journal of Pharmacology*. *British Journal of Pharmacology*, *175*, 407–411. https://doi.org/10.1111/bph.14112

Angulo, J., Cuevas, P., Gabancho, S., Gonzalez-Corrochano, R., Videla, S., & Saenz de Tejada, I. (2005). Enhancement of both EDHF and NO/cGMP pathways is necessary to reverse erectile dysfunction in diabetic rats. *The Journal of Sexual Medicine*, *2*(3), 341–346. https://doi.org/10.1111/j.1743-6109.2005.20348.x

14765381, 2025, 15, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.70034 by Universidad Complutense De Madrid, Wiley Online Library on [12/01/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Angulo, J., El Assar, M., Sevilleja-Ortiz, A., Fernandez, A., Sanchez-Ferrer, A., Romero-Otero, J., Martinez-Salamanca, J. I., La Fuente, J. M., & Rodriguez-Manas, L. (2019). Short-term pharmacological activation of Nrf2 ameliorates vascular dysfunction in aged rats and in pathological human vasculature. A potential target for therapeutic intervention. *Redox Biology*, *26*, 101271. https://doi.org/10.1016/j.redox.2019.101271

Angulo, J., Gonzalez-Corrochano, R., Cuevas, P., Fernandez, A., La Fuente, J. M., Rolo, F., Allona, A., & Saenz de Tejada, I. (2010). Diabetes exacerbates the functional deficiency of NO/cGMP pathway associated with erectile dysfunction in human corpus cavernosum and penile arteries. *The Journal of Sexual Medicine*, *7*(2 Pt 1), 758–768. https://doi.org/10.1111/j.1743-6109.2009.01587.x

Anilkumar, S. A., Dutta, S., Aboo, S., & Ismail, A. (2024). Vitamin D as a modulator of molecular pathways involved in CVDs: Evidence from preclinical studies. *Life Sciences*, *357*, 123062. https://doi.org/10.1016/j.lfs.2024.123062

Callejo, M., Blanco, I., Barbera, J. A., & Perez-Vizcaino, F. (2021). Vitamin D deficiency, a potential cause for insufficient response to sildenafil in pulmonary arterial hypertension. *The European Respiratory Journal*, *58*(5), 2101204. https://doi.org/10.1183/13993003.01204-2021

Callejo, M., Morales-Cano, D., Olivencia, M. A., Mondejar-Parreno, G., Barreira, B., Tura-Ceide, O., Martinez, V. G., Serrano-Navarro, A., Moreno, L., Morrell, N., Perros, F., Vicente, A., Cogolludo, A., & Perez-Vizcaino, F. (2024). Vitamin D receptor and its antiproliferative effect in human pulmonary arterial hypertension. *Scientific Reports*, *14*(1), 27445. https://doi.org/10.1038/s41598-024-78380-9

Canguven, O., & Al Malki, A. H. (2021). Vitamin D and male erectile function: An updated review. *The World Journal of Men's Health*, *39*(1), 31–37. https://doi.org/10.5534/wjmh.190151

Canguven, O., Lagoda, G., Sezen, S. F., & Burnett, A. L. (2009). Losartan preserves erectile function after bilateral cavernous nerve injury via antifibrotic mechanisms in male rats. *The Journal of Urology*, *181*(6), 2816–2822. https://doi.org/10.1016/j.juro.2009.01.097

Carlberg, C., & Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor. *Steroids*, *78*(2), 127–136. https://doi.org/10.1016/j.steroids.2012.10.019

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*, 3907–3913. https://doi.org/10.1111/bph.15868

Demirci, A., Cakan, M., & Topcuoglu, M. (2021). Whether adding vitamin D to tadalafil 5 mg treatment is useful in patients with erectile dysfunction and vitamin D deficiency? *Urologia Internationalis*, *105*(5–6), 514–519. https://doi.org/10.1159/000514056

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., Leblanc, M., Coulter, S., He, M., Scott, C., Lau, S. L., Atkins, A. R., Barish, G. D., Gunton, J. E., Liddle, C., Downes, M., & Evans, R. M. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell*, *153*(3), 601–613. https://doi.org/10.1016/j.cell.2013.03.028

Duarte Romero, B., Waterhouse, M., Baxter, C., McLeod, D. S. A., English, D. R., Armstrong, B. K., Chung, E., Ebeling, P. R., Hartel, G., van der Pols, J. C., Roberts, M., Venn, A. J., Webb, P. M., Whiteman, D. C., & Neale, R. E. (2024). The effect of three years of vitamin D supplementation on erectile dysfunction: Results from the randomized placebo-controlled D-Health Trial. *Clinical Nutrition ESPEN*, *60*, 109–115. https://doi.org/10.1016/j.clnesp.2024.01.011

Dusso, A. S., Brown, A. J., & Slatopolsky, E. (2005). Vitamin D. *American Journal of Physiology. Renal Physiology*, *289*(1), F8–F28. https://doi.org/10.1152/ajprenal.00336.2004

Gallagher, J. C., & Rosen, C. J. (2023). Vitamin D: 100 years of discoveries, yet controversy continues. *The Lancet Diabetes and Endocrinology*, *11*(5), 362–374. https://doi.org/10.1016/S2213-8587(23)00060-8

Gipson, T. S., Bless, N. M., Shanley, T. P., Crouch, L. D., Bleavins, M. R., Younkin, E. M., Sarma, V., Gibbs, D. F., Tefera, W., McConnell, P. C., Mueller, W. T., Johnson, K. J., & Ward, P. A. (1999). Regulatory effects of endogenous protease inhibitors in acute lung inflammatory injury. *Journal of Immunology*, *162*(6), 3653–3662. https://doi.org/10.4049/jimmunol.162.6.3653

Gratzke, C., Angulo, J., Chitaley, K., Dai, Y. T., Kim, N. N., Paick, J. S., Simonsen, U., Uckert, S., Wespes, E., Andersson, K. E., Lue, T. F., & Stief, C. G. (2010). Anatomy, physiology, and pathophysiology of erectile dysfunction. *The Journal of Sexual Medicine*, *7*(1 Pt 2), 445–475. https://doi.org/10.1111/j.1743-6109.2009.01624.x

Han, H., Chung, S. I., Park, H. J., Oh, E. Y., Kim, S. R., Park, K. H., Lee, J. H., & Park, J. W. (2021). Obesity-induced vitamin D deficiency contributes to lung fibrosis and airway hyperresponsiveness. *American Journal of Respiratory Cell and Molecular Biology*, *64*(3), 357–367. https://doi.org/10.1165/rcmb.2020-0086OC

Harada, N., Ito, K., Hosoya, T., Mimura, J., Maruyama, A., Noguchi, N., Yagami, K., Morito, N., Takahashi, S., Maher, J. M., Yamamoto, M., & Itoh, K. (2012). Nrf2 in bone marrow-derived cells positively contributes to the advanced stage of atherosclerotic plaque formation. *Free Radical Biology & Medicine*, *53*(12), 2256–2262. https://doi.org/10.1016/j.freeradbiomed.2012.10.001

Henriksen, P. A., Hitt, M., Xing, Z., Wang, J., Haslett, C., Riemersma, R. A., Webb, D. J., Kotelevtsev, Y. V., & Sallenave, J. M. (2004). Adenoviral gene delivery of elafin and secretory leukocyte protease inhibitor attenuates NF-κB-dependent inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli. *Journal of Immunology*, *172*(7), 4535–4544. https://doi.org/10.4049/jimmunol.172.7.4535

Horsanali, M. O., Eren, H., Dil, E., Caglayan, A., Erdogan, O., & Ekren, F. (2020). Novel predictive risk factor of erectile dysfunction: Serum 25-hydroxy vitamin D. *Andrologia*, *52*(10), e13767. https://doi.org/10.1111/and.13767

Inman, B. A., Sauver, J. L., Jacobson, D. J., McGree, M. E., Nehra, A., Lieber, M. M., Roger, V. L., & Jacobsen, S. J. (2009). A population-based, longitudinal study of erectile dysfunction and future coronary artery disease. *Mayo Clinic Proceedings*, *84*(2), 108–113. https://doi.org/10.4065/84.2.108

Jaumann, F., Elssner, A., Mazur, G., Dobmann, S., & Vogelmeier, C. (2000). Transforming growth factor-beta1 is a potent inhibitor of secretory leukoprotease inhibitor expression in a bronchial epithelial cell line. Munich Lung Transplant Group. *The European Respiratory Journal*, *15*(6), 1052–1057. https://doi.org/10.1034/j.1399-3003.2000.01513.x

Jeremy, J. Y., Jones, R. A., Koupparis, A. J., Hotston, M., Persad, R., Angelini, G. D., & Shukla, N. (2007). Reactive oxygen species and erectile dysfunction: Possible role of NADPH oxidase. *International Journal of Impotence Research*, *19*(3), 265–280. https://doi.org/10.1038/sj.ijir.3901523

Kessler, A., Sollie, S., Challacombe, B., Briggs, K., & Van Hemelrijck, M. (2019). The global prevalence of erectile dysfunction: A review. *BJU International*, *124*(4), 587–599. https://doi.org/10.1111/bju.14813

Kongpol, K., Nernpermpisooth, N., Prompunt, E., & Kumphune, S. (2019). Endothelial-cell-derived human secretory leukocyte protease inhibitor (SLPI) protects cardiomyocytes against ischemia/reperfusion injury. *Biomolecules*, *9*(11), 678. https://doi.org/10.3390/biom9110678

Kozin, S. V., Maimon, N., Wang, R., Gupta, N., Munn, L., Jain, R. K., & Garkavtsev, I. (2017). Secretory leukocyte protease inhibitor (SLPI) as a potential target for inhibiting metastasis of triple-negative breast cancers. *Oncotarget*, *8*(65), 108292–108302. https://doi.org/10.18632/oncotarget.22660

14765381, 2025, 15, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.70034 by Universidad Complutense De Madrid, Wiley Online Library on [12/01/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Li, J., Wang, J. J., & Zhang, S. X. (2015). NADPH oxidase 4-derived H_2O_2 promotes aberrant retinal neovascularization via activation of VEGF receptor 2 pathway in oxygen-induced retinopathy. *Journal Diabetes Research*, *2015*, 963289. https://doi.org/10.1155/2015/963289

Li, M., Zhuan, L., Wang, T., Rao, K., Yang, J., Yang, J., Quan, W., Liu, J., & Ye, Z. (2012). Apocynin improves erectile function in diabetic rats through regulation of NADPH oxidase expression. *The Journal of Sexual Medicine*, *9*(12), 3041–3050. https://doi.org/10.1111/j.1743-6109.2012.02960.x

Li, Y. C., Amling, M., Pirro, A. E., Priemel, M., Meuse, J., Baron, R., Delling, G., & Demay, M. B. (1998). Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. *Endocrinology*, *139*(10), 4391–4396. https://doi.org/10.1210/endo.139.10.6262

Li, Y. C., Pirro, A. E., Amling, M., Delling, G., Baron, R., Bronson, R., & Demay, M. B. (1997). Targeted ablation of the vitamin D receptor: An animal model of vitamin D-dependent rickets type II with alopecia. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(18), 9831–9835. https://doi.org/10.1073/pnas.94.18.9831

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

May, H. T., Bair, T. L., Lappe, D. L., Anderson, J. L., Horne, B. D., Carlquist, J. F., & Muhlestein, J. B. (2010). Association of vitamin D levels with incident depression among a general cardiovascular population. *American Heart Journal*, *159*(6), 1037–1043. https://doi.org/10.1016/j.ahj.2010.03.017

Mitidieri, E., Cirino, G., d'Emmanuele di Villa Bianca, R., & Sorrentino, R. (2020). Pharmacology and perspectives in erectile dysfunction in man. *Pharmacology & Therapeutics*, *208*, 107493. https://doi.org/10.1016/j.pharmthera.2020.107493

Montenegro, K. R., Cruzat, V., Carlessi, R., & Newsholme, P. (2019). Mechanisms of vitamin D action in skeletal muscle. *Nutrition Research Reviews*, *32*(2), 192–204. https://doi.org/10.1017/S0954422419000064

Murad, F., Mittal, C. K., Arnold, W. P., Katsuki, S., & Kimura, H. (1978). Guanylate cyclase: Activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Advances in Cyclic Nucleotide Research*, *9*, 145–158.

Nernpermpisooth, N., Prompunt, E., & Kumphune, S. (2017). An in vitro endothelial cell protective effect of secretory leukocyte protease inhibitor against simulated ischaemia/reperfusion injury. *Experimental and Therapeutic Medicine*, *14*(6), 5793–5800. https://doi.org/10.3892/etm.2017.5272

Olivencia, M. A., Esquivel-Ruiz, S., Callejo, M., Mondejar-Parreno, G., Quintana-Villamandos, B., Barreira, B., Sacedon, R., Cogolludo, A., Perros, F., Mendes-Ferreira, P., & Perez Vizcaino, F. (2022). Cardiac and pulmonary vascular dysfunction in vitamin D-deficient *Bmpr2*-mutant rats. *American Journal of Respiratory Cell and Molecular Biology*, *67*(3), 402–405. https://doi.org/10.1165/rcmb.2022-0001LE

Olivencia, M. A., Gil de Biedma-Elduayen, L., Gimenez-Gomez, P., Barreira, B., Fernandez, A., Angulo, J., Colado, M. I., O'Shea, E., & Perez-Vizcaino, F. (2023). Oxidized soluble guanylyl cyclase causes erectile dysfunction in alcoholic mice. *British Journal of Pharmacology*, *180*(18), 2361–2376. https://doi.org/10.1111/bph.16087

Park, N. C., Kim, T. N., & Park, H. J. (2013). Treatment strategy for non-responders to PDE5 inhibitors. *The World Journal of Men's Health*, *31*(1), 31–35. https://doi.org/10.5534/wjmh.2013.31.1.31

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Wurbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Ramasamy, I. (2020). Vitamin D metabolism and guidelines for vitamin D supplementation. *Clinical Biochemist Reviews*, *41*(3), 103–126. https://doi.org/10.33176/AACB-20-00006

Roth, D. E., Abrams, S. A., Aloia, J., Bergeron, G., Bourassa, M. W., Brown, K. H., Calvo, M. S., Cashman, K. D., Combs, G., De-Regil, L. M., Jefferds, M. E., Jones, K. S., Kapner, H., Martineau, A. R., Neufeld, L. M., Schleicher, R. L., Thacher, T. D., & Whiting, S. J. (2018). Global prevalence and disease burden of vitamin D deficiency: A roadmap for action in low- and middle-income countries. *Annals of the new York Academy of Sciences*, *1430*(1), 44–79. https://doi.org/10.1111/nyas.13968

Schafer, M., Farwanah, H., Willrodt, A. H., Huebner, A. J., Sandhoff, K., Roop, D., Hohl, D., Bloch, W., & Werner, S. (2012). Nrf2 links epidermal barrier function with antioxidant defense. *EMBO Molecular Medicine*, *4*(5), 364–379. https://doi.org/10.1002/emmm.201200219

Shamloul, R., & Ghanem, H. (2013). Erectile dysfunction. *Lancet*, *381*(9861), 153–165. https://doi.org/10.1016/S0140-6736(12)60520-0

Tarroni, P., Villa, I., Mrak, E., Zolezzi, F., Mattioli, M., Gattuso, C., & Rubinacci, A. (2012). Microarray analysis of $1,25(OH)_2D_3$ regulated gene expression in human primary osteoblasts. *Journal of Cellular Biochemistry*, *113*(2), 640–649. https://doi.org/10.1002/jcb.23392

Troidl, K., Ruding, I., Cai, W. J., Mucke, Y., Grossekettler, L., Piotrowska, I., Apfelbeck, H., Schierling, W., Volger, O. L., Horrevoets, A. J., Grote, K., Schmitz-Rixen, T., Schaper, W., & Troidl, C. (2009). Actin-binding rho activating protein (*Abra*) is essential for fluid shear stress-induced arteriogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *29*(12), 2093–2101. https://doi.org/10.1161/ATVBAHA.109.195305

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., Bourdeau, V., Konstorum, A., Lallemant, B., Zhang, R., Mader, S., & White, J. H. (2005). Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D_3 target genes. *Molecular Endocrinology*, *19*(11), 2685–2695. https://doi.org/10.1210/me.2005-0106

Wehr, E., Pilz, S., Boehm, B. O., Marz, W., & Obermayer-Pietsch, B. (2010). Association of vitamin D status with serum androgen levels in men. *Clinical Endocrinology*, *73*(2), 243–248. https://doi.org/10.1111/j.1365-2265.2009.03777.x

Wu-Wong, J. R., Li, X., & Chen, Y. W. (2015). Different vitamin D receptor agonists exhibit differential effects on endothelial function and aortic gene expression in 5/6 nephrectomized rats. *The Journal of Steroid Biochemistry and Molecular Biology*, *148*, 202–209. https://doi.org/10.1016/j.jsbmb.2014.12.002

Yafi, F. A., Jenkins, L., Albersen, M., Corona, G., Isidori, A. M., Goldfarb, S., Maggi, M., Nelson, C. J., Parish, S., Salonia, A., Tan, R., Mulhall, J. P., & Hellstrom, W. J. (2016). Erectile dysfunction. *Nature Reviews. Disease Primers*, *2*, 16003. https://doi.org/10.1038/nrdp.2016.3

Zhang, L. W., Piao, S., Choi, M. J., Shin, H. Y., Jin, H. R., Kim, W. J., Song, S. U., Han, J. Y., Park, S. H., Mamura, M., Kim, S. J., Ryu, J. K., & Suh, J. K. (2008). Role of increased penile expression of transforming growth factor-β1 and activation of the Smad signaling pathway in erectile dysfunction in streptozotocin-induced diabetic rats. *The Journal of Sexual Medicine*, *5*(10), 2318–2329. https://doi.org/10.1111/j.1743-6109.2008.00977.x

Zhang, Z. H., Luo, B., Xu, S., Zhang, Z. C., Xing, W. Y., Chen, Y. H., Zhang, C., Wang, H., Xie, D. D., & Xu, D. X. (2021). Long-term vitamin

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D deficiency promotes renal fibrosis and functional impairment in middle-aged male mice. *The British Journal of Nutrition*, *125*(8), 841–850. https://doi.org/10.1017/S0007114520003232

Zhao, L., Han, S., Su, H., Li, J., Zhi, E., Li, P., Yao, C., Tian, R., Chen, H., Chen, H., Luo, J., Shi, C., Ji, Z., Hu, J., Wu, G., Zhou, W., Tang, Y., Chen, Y., Lin, G., … Li, Z. (2022). Single-cell transcriptome atlas of the human corpus cavernosum. *Nature Communications*, *13*(1), 4302. https://doi.org/10.1038/s41467-022-31950-9

Zhong, Q. Q., Wang, X., Li, Y. F., Peng, L. J., & Jiang, Z. S. (2017). Secretory leukocyte protease inhibitor promising protective roles in obesity-associated atherosclerosis. *Experimental Biology and Medicine*, *242*(3), 250–257. https://doi.org/10.1177/1535370216672747

Zhu, S., Xiong, Y., Yu, B., Wang, H., Zhang, F., Wu, C., Qin, F., & Yuan, J. (2023). Vitamin D3 improved erectile function recovery by regulating autophagy and apoptosis in a rat model of cavernous nerve injury. *International Journal of Impotence Research*, *36*, 430–436. https://doi.org/10.1038/s41443-023-00679-4

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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14765381, 2025, 15, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.70034 by Universidad Complutense De Madrid, Wiley Online Library on [12/01/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License