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Clinical Research Article

Effects of Sex Hormones on Vascular Reactivity in Boys With Hypospadias

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Abstract

Background: Arteries from boys with hypospadias demonstrate hypercontractility and impaired vasorelaxation. The role of sex hormones in these responses in unclear.

Aims: We compared effects of sex steroids on vascular reactivity in healthy boys and boys with hypospadias.

Methods: Excess foreskin tissue was obtained from 11 boys undergoing hypospadias repair (cases) and 12 undergoing routine circumcision (controls) (median age [range], 1.5 [1.2-2.7] years) and small resistance arteries were isolated. Vessels were mounted on wire myographs and vascular reactivity was assessed in the absence/presence of 17β-estradiol, dihydrotestosterone (DHT), and testosterone.

Results: In controls, testosterone and 17β-estradiol increased contraction (percent of maximum contraction [Emax]: 83.74 basal vs 125.4 after testosterone, $P < .0002$; and 83.74 vs 110.2 after estradiol, $P = .02$). 17β-estradiol reduced vasorelaxation in arteries from controls (Emax: 10.6 vs 15.6 to acetylcholine, $P < .0001$; and Emax: 14.6 vs 20.5 to sodium nitroprusside, $P < .0001$). In hypospadias, testosterone (Emax: 137.9 vs 107.2, $P = .01$) and 17β-estradiol (Emax: 156.9 vs 23.6, $P < .0001$) reduced contraction. Androgens, but not 17β-estradiol, increased endothelium-dependent and endothelium-independent vasorelaxation in cases (Emax: 77.3 vs 51.7 with testosterone, $P = .02$; and vs 48.2 with DHT to acetylcholine, $P = .0001$; Emax: 43.0 vs 39.5 with testosterone, $P = .02$; and 39.6 vs 37.5 with DHT to sodium nitroprusside, $P = .04$).

Conclusion: In healthy boys, testosterone and 17β-estradiol promote a vasoconstrictor phenotype, whereas in boys with hypospadias, these sex hormones reduce vasoconstriction, with androgens promoting vasorelaxation. Differences in baseline artery function may therefore be sex hormone-independent and the impact of early-life variations in androgen exposure on vascular function needs further study.

Key Words: testosterone, vessel, estrogen, dihydrotestosterone, androgen

Abbreviations: DHT, dihydrotestosterone; Emax, percent of maximal contraction; ESR, estrogen receptor; GPER, G protein-coupled estrogen receptor; KCl, potassium chloride; MPW, masculinization programming window; VSMC, vascular smooth muscle cell.

Hypospadias, a condition that is associated with inadequate development of the urethra and a meatus on the ventral aspect of the penile shaft, is often associated with reduced testosterone synthesis or action. Masculinization of the undifferentiated external genitalia is induced by testosterone produced by the fetal testes between weeks 9 and 20 of gestation (1, 2), which is often referred to as the masculinization programming window (MPW) (2-5). Reduced exposure to testosterone during this period may be associated with abnormalities of the external genitalia, such as hypospadias. However, in most cases, boys with hypospadias will have normal endocrine function in childhood (6). Regardless of any endocrine abnormality, hypospadias is also associated with shorter anogenital distance and anoscrotal distance, indicators of reduced antenatal androgen exposure (3). Thus, despite being associated with normal postnatal gonadal function, it is possible that hypospadias may represent a short period of inadequate androgen exposure during a critical period of fetal programming.

Recently, vascular reactivity in subcutaneous resistance arteries from boys with hypospadias and healthy control boys demonstrated altered vascular reactivity with hypercontractility and reduced endothelium-dependent and -independent vasorelaxation compared with arteries from controls (7). The vascular system is the first major system to function in the embryo, appearing in the middle of the third week of

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gestation (1). Vascular smooth muscle cells (VSMCs) have been localized in the blood vessels of embryos from 7 weeks' gestation (8) and major vasculogenesis is, therefore, being undertaken at the same time as the MPW. Impaired vascular reactivity and increased arterial stiffness have been associated with placental insufficiency (9), although there are conflicting data about whether adverse fetal programming can influence arterial health clinically in children (10).

Sexual dimorphism is evident in several important physiological mechanisms for the development of cardiovascular disease, including differences in angiotensin receptor expression (11), Ca2 + signalling (12), and oxidative stress generation (13). Although the underlying reasons why females and males should have these differences have not yet been fully elucidated, it stands to reason that these differences may be mediated by sex hormones, given that both testosterone and estradiol are vasoactive hormones. Testosterone has been reported to alter vascular reactivity via several mechanisms including the regulation of Ca^{2+} influx and Ca^{2+} channel expression (14), the induction of reactive oxygen species generation secondary to stimulation of nicotinamide adenine dinucleotide phosphate oxidases (15), amplification of Rho kinase signalling pathways (16), and the regulation of thromboxane A2 receptors (17). Meanwhile, estrogen is also known to affect the vasculature, classically acting through the estrogen receptors estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2), both of which have been identified in endothelial and VSMCs and have been implicated in the regulation of endothelial function and vasodilation (18). In addition, estrogen can act through the G protein-coupled estrogen receptor (GPER), resulting in rapid increases in endothelial nitric oxide synthase and production of nitric oxide (19).

Given that hypospadias represents an abnormality of penile development that is related to disrupted androgen exposure during the MPW, it is of interest to determine the effects of sex steroids on arteries from boys with this condition. Aberrant estrogen signalling and endocrine disruptors affecting the estrogen receptors have also been associated in the etiology of hypospadias (20, 21). As such, the role of estrogen on these arteries should also be defined.

The aim of this study was therefore to identify whether androgens or estrogen alter vascular reactivity in arteries from boys with hypospadias.

Methods

Patients

Parents of boys undergoing hypospadias repair and routine circumcision from 2016 to 2019 at a tertiary pediatric surgical center in the West of Scotland were approached and written informed consent for the study was obtained. In total, 11 boys with hypospadias and 12 boys undergoing circumcision were recruited.

Hormone Analysis

Where consent was obtained, at the time of IV cannulation before anesthetic administration, 2 to 3 mL of blood was obtained for plasma testosterone, anti-Müllerian hormone, FSH, LH, and estradiol levels as well as fasting cholesterol, triglycerides and glucose, which were analyzed in the local National Health Service laboratory according to standard protocols (Table 1).

Genetic Analysis

DNA was extracted from blood samples per standard practice for patients undergoing routine clinical genetic testing. Chromosomal abnormality was identified by karyotype or array-comparative genome hybridization. Analysis of genes associated with differences of sex development (Table 2) was performed using a custom-designed Agilent SureSelectQXT hybridization panel performed on the Illumina MiSeq and analyzed using QIAGEN Biomedical workbench, Golden Helix VarSeq, and Alamut. Pathogenicity of detected variants was determined using Alamut Visual version 2.11 (Interactive Biosoftware, Rouen, France) and discussed at a multidisciplinary diagnostic team meeting. Coverage of the panel is a minimum of 99.55% at >30× with sensitivity 96.97% (95% confidence interval).

Vascular Reactivity

Excess skin, which was removed as part of the standard surgical procedure was stored in nonsterile PBS (Gibco Life Technologies, UK) on ice before dissection. Subcutaneous resistance arteries were dissected from the skin and 1.5- to 2-mm lengths of arteries were mounted onto wire myographs (AD Instruments, UK) for vascular reactivity studies as previously described (7, 22). For assessment of the effects of sex steroids,

Table 1. Details of biochemical analyses

Assay	Equipment	Functional sensitivity	Coefficient of variation (95% CI)	RRID
AMH	Beckman Access MDL assay (Beckman Coulter, USA)	1 pmol/L	<5%	AB_2892998
Cholesterol	Abbott Architect, enzymatic method (BioRad, USA)	0.17 mmol/L	<2%	Enzymatic assay—no antibodies used
FSH	Abbott Architect, one step assay (BioRad, USA)	0.1 IU/L	<5%	AB_2813910
Glucose	Abbott Architect, enzymatic method (BioRad, USA)	0.28 mmol/L	<2%	Enzymatic assay—no antibodies used
LH	Abbott Architect, one step assay (BioRad, USA)	0.1 IU/L	<5%	AB_2813909
Estradiol	Abbott Architect, one-step assay (BioRad, USA)	37 pmol/L	<8%	AB_2813911
Testosterone	Xevo TQS® Tandem Mass Spectrometer, LC/MS (Waters Corporation, USA)	0.5 nmol/L	<8%	No enzyme or antibodies used
Triglycerides	Abbott Architect, enzymatic method (BioRad, USA)	0.08 mmol/L	<1%	Enzymatic assay—no antibodies used

Abbreviations: AMH, anti-Müllerian hormone; LC/MS, liquid chromatography/mass spectrometry; RRID, Research Resource Identifier

Table 2. Genes investigated using the local disorders of sex development gene panel

AMH	AMHR2	ANOS1	AR	ARX	ATRX	CBX2	CHD7	CUL4B
CYB5A	CYP11A1	CYP11B1	CYP17A1	CYP19A1	DHCR7	DHH	DMRT1	FEZF1
FGF8	FGFR1	FOXL2	FSHB	GATA4	GNRH1	GNRHR	HSD17B3	HSD3B2
HSD3B2	INSL3	KISS1R	LHB	LHCGR	MAMLD1	MAP3K1	NR0B1	NR3C1
NR5A1	POR	PROK2	PROK2R	RESP01	RXFP2	SEMA3E	SOX2	SOX3
SOX9	SOX10	SPRY4	SRD5A2	SRY	STAR	TAC3	TACR3	TSPYL1
WDR11	WNT4	WT1						

arteries were incubated for 30 minutes with 1×10^{-7} M testosterone (Sigma Aldrich, UK), 1×10^{-9} M 17β-estradiol (Sigma Aldrich, UK), 1×10^{-9} M dihydrotestosterone (Sigma Aldrich, UK). or vehicle before vascular reactivity curves. The vascular reactivity curve was undertaken and the vessel was washed before the next curve and then reincubated with the steroid again for 30 minutes. The doses for the sex steroids were chosen because they had previously been investigated using dose-response curves by members of the same laboratory group (23, 24) and were in line with those reported in vascular tissue in the recent scientific literature (25, 26). Importantly, a literature review before the experiments beginning suggested that higher doses of estrogen and dihydrotestosterone (DHT) in particular could inhibit VSMC proliferation and action (27).

Constriction was investigated using cumulative increasing doses of the thromboxane A2 analogue U46619 (Enzo Life Sciences, UK) (1×10^{-10}-3×10^{-6} M). Endothelium-dependent relaxation was investigated using relaxation curves to acetylcholine (Sigma Aldrich, UK) (1×10^{-9}-3×10^{-5} M) and endothelium-independent vasodilation was investigated using sodium nitroprusside (Honeywell, UK) (1×10^{-9}-3×10^{-5} M). The starting dose of the constrictor/dilator was added and the next dose was not added until the vessel reached maximal contraction/dilation. This occurred at times ranging from 30 seconds to 5 minutes. At each dose, the maximal contraction/dilation was measured by the AD Instruments software.

Different arteries were used for different sex steroid incubations to prevent contamination of the artery and potential confounding of the results. On average, 4 to 8 arteries were dissected per child but not all were functional (defined as ability to contract in response to 62.5 mM potassium chloride [KCl]); therefore, not all incubations and vascular reactivity curves were possible from every child. Where able, 2 arteries were used for each condition (control, testosterone, DHT, and 17β-estradiol) from each child, to allow for a mean to be taken of the results to ensure improved reliability and reproducibility, but if this was not possible then 1 artery was used for the different incubations. All 3 vascular reactivity curves were performed on each artery. Where the same condition was used more than once in the same child, the mean of the results was used for analysis. Results are shown as % of maximal contraction (Emax) to 62.5 mM KCl. Magnitude of physiological saline solution (0.25 M NaCl, 0.001 M KCl, 50 mM $NAHC0_3$, 2 mM KH_2PO_4, 1 mM glucose, 2.5 mM $CaCl_2$) plus 62.5 mM KCl response was taken at the beginning of the experiment in the presence and absence of the hormones after 30 minutes' incubation before vascular reactivity curves.

Culture of VSMCs From Small Arteries From Young Boys

Resistance arteries from the penile skin were cleaned of connective tissue and pooled in a sterile Eppendorf tube. VSMCs were dissociated using an enzymatic digestion mix at 37 °C using gentle agitation. The cell suspension was then centrifuged and resuspended in complete F12 medium (Sigma Aldrich, UK). Within 48 hours the medium was replaced with complete VSMC medium (231 medium; Invitrogen, UK; 25 mL Smooth Muscle Growth Supplement; Invitrogen, UK; 5 mL penicillin/streptomycin). The cells were then grown until confluence in flasks and split using trypsin until passages 4 through 6. Once confluent, VSMCs were rendered quiescent with serum deprivation (5% Smooth Muscle Growth Supplement in 500 mL VSMC medium 231) overnight and then incubated with either drugs or 5 µL of vehicle depending on the specific protocol used. After stimulation, plates were washed with ice cold nonsterile PBS and stored in −20 °C until use.

Quantitative Real-time PCR

mRNA expression was measured on VSMCs using quantitative real-time PCR for AR, ESR1, ESR2, and GPER (Table 3). Total RNA was extracted using Qiazol (Qiagen, UK). Target gene expression was identified using Qiagen QuantiTech primer assays (Qiagen, UK) and SYBR Green (UK). Transcript gene expression was normalized using the housekeeping gene. The $2^{\delta\delta CT}$ method was then used to calculate relative gene expression. Results are expressed as % control.

Immunoblotting

Immunoblots were performed as previously described (7). Membranes were incubated with the primary antibody (androgen receptor, details in Table 4) overnight at 4 °C. Thereafter, they were washed 3× in Tris buffered saline-Tween 20. Membranes were then incubated with secondary antibodies for 1 hour at room temperature and fluorescent signals measured using the Odyssey CLX, LI-COR (LI-COR Biosciences, UK) scanner and analyzed using ImageStudioLite (LI-COR Biosciences, UK) software. Alpha tubulin (Sigma Aldrich, UK; 1:10,000, 3% BSA/Tris buffered saline-Tween 20) was used on all membranes as an internal housekeeping loading control protein. Results were normalized according to cellular protein levels. Results are expressed in arbitrary units.

Calcium Signalling

Fluorescent measurement of the Ca^{2+} indicator Cal-520 acetoxymethyl ester (Cal-520/AM, Abcam, 10 µmol/L) in response to U46619 (10^{-6} M) was used to identify differences

Table 3. Primers for mRNA expression

Gene	Forward primer	Reverse primer
Androgen receptor (AR)	5′ -CCT GGC TTC CGC AAC TTA CAC-3′	5′ -GGA CTT GTG CAT GCG GTA CTC A- 3′
Estrogen receptor 1 (ESR1)	5′ -CCA CCA ACC AGT GCA CCA TT-3′	5′ -GGT CTT TTC GTA TCC CAC CTT TC-3′
Estrogen receptor 1 (ESR2)	5′ -AGA GTC CCT GGT GTG AAG CAA G-3′	5′ -GAC AGC GCA GAA GTG AGC ATC-3′
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5′—GAG TCA ACG GAT TTG GTC GT—3′	5′- TTG ATT TTG GAG GGA TCT CG—3′
G protein-coupled estrogen receptor (GPER)	5′ -CTG CAC GAG CGG TAC TAC GA- 3′	5′ -CAG ATG AGG CCA CAG CTC AG- 3′

Table 4. Antibodies for protein expression

Name	Manufacturer	Dilution	Host species	RRID
Alpha tubulin	Abcam, UK	1:10 000	Rabbit	AB_942168
Androgen Receptor	Sigma Aldrich, UK	1:1000	Rabbit	AB_262132

Abbreviation: RRID, Research Resource Identifier.

in Ca^{2+} using an inverted epifluorescence microscope (Zeiss, UK) with excitatory wavelengths of 490 nm and emission at 535 nm. Results are expressed as area under the curve.

Statistical Analysis

Mean ± SD was calculated for all data. Normality of all data was assessed using the Shapiro-Wilks test. Statistical outliers were removed using the ROUT method. Best fit cumulative concentration curves were compared with the extra sum-of-squares *F* test. Differences between groups were calculated using 1-way ANOVA followed by Tukey posttest or Student *t* tests when appropriate. Analyses were done to compare differences between basal levels and stimulated levels for the same group of participants as well as differences between controls and boys with hypospadias. For all analyses, a P value <.05 was deemed statistically significant. All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc, USA).

Ethics

This study was authorized by the West of Scotland Research Ethics Committee (REC reference: 16/WS/0186) and by the West of Scotland Research and Development Office (study reference GN16CA519).

Results

Participants

Eleven boys with hypospadias and 12 boys undergoing routine circumcision were recruited. All had isolated distal hypospadias. The median (range) age of the boys was 1.5 years (1.2-2.7). The median gestation at birth was 40 weeks (37-41) and their median birthweight was 3.5 kg (3.0-3.9). None of the patients were on any medication. Except for 1 control boy who had a history of bilateral hydronephrosis, none of the boys had any significant medical history. All boys received caudal analgesia for the procedure and none had received prior treatment with androgens or estrogens. None of the boys had any variants detected on gene panel testing for differences of sex development. For comparison, 12 control boys were included in this study, with a median (range) age of 1.8 years (1.2-2.8). Their median gestation at birth was 40 weeks (36-41) and their median birthweight was 3.5 kg (2.2-4.1). Boys with hypospadias had increased triglycerides compared with controls but there were no other differences in baseline biochemistry (Table 5). The number of arteries incubated from each patient per condition is shown in Table 6.

The Effects of Sex Steroids on Arteries From Healthy Boys

In arteries from controls, testosterone increased contraction (Emax: 83.7 ± 9.2 basal vs 125.4 ± 5.1, $P < .0002$). 17β-estradiol also increased contraction (Emax: 83.7 ± 9.2 basal vs 110.2 ± 10.5; $P = .02$). DHT had no effect on contraction (Fig. 1A). Testosterone and DHT had no effect on endothelium-dependent vasorelaxation in controls. However, 17β-estradiol reduced endothelium-dependent vasorelaxation in arteries from controls (Emax: 10.6 ± 7.7 vs 15.6 ± 2.3, $P < .0001$) (Fig. 1B). Testosterone and DHT had no effect on endothelium-independent relaxation in control arteries. However, 17β-estradiol reduced endothelium-independent vasorelaxation (Emax: 14.6 ± 7.7 vs 20.5 ± 2.3, $P < .0001$) (Fig. 1C). Testosterone and DHT had no effect on Ca^{2+} influx in VSMCs from controls (Fig. 1D-E). 17β-estradiol increased Ca^{2+} influx in VSMCs from controls ($P = .0005$) (Fig. 1F-G).

The Effects of sex Steroids on Arteries From Boys With Hypospadias

In arteries from boys with hypospadias, testosterone reduced contraction (Emax: 137.9 ± 5.1 basal vs 107.2 ± 5.8, $P = .01$), although 17β-estradiol reduced contraction to an even greater extent (Emax: 137.9 ± 5.1 basal vs 23.6 ± 3.3, $P < .0001$). DHT had no effect on contraction in arteries from boys with hypospadias (Fig. 2A). Testosterone and DHT increased endothelium-dependent vasorelaxation in arteries from boys with hypospadias (Emax: 77.3 ± 4.5 vs 51.7 ± 5.7, $P = .02$; and vs 48.2 ± 10.7, $P = .0001$) (Fig. 2B). Testosterone and DHT also increased endothelium-independent vasorelaxation (Emax: 43.0 ± 4.0 vs 39.5 ± 3.6, $P = .02$; and vs 37.5 ± 5.1, $P = .04$) 17β-estradiol had no effect on either endothelium-dependent or endothelium-independent vasorelaxation (Fig. 2C). Testosterone reduced Ca^{2+} influx in boys with hypospadias ($P = .03$) (Fig. 2D-E). DHT had no effect on Ca^{2+} influx in VSMCs from boys with hypospadias (Fig. 2D-E). 17β-estradiol reduced Ca^{2+} influx in VSMCs from cases ($P = .02$) (Fig. 2F-G).

Table 5. Fasting blood levels of participants in the study on the day of hypospadias repair (cases) or circumcision (controls)

Median (range)	Cases n = 11	Controls n = 12	*P*
AMH, pmol/L	875 (421-1350)	987 (371-1725)	.98
Cholesterol, mmol/L	3.8 (3.2-4.9)	3.9 (2-4.7)	.29
FSH, U/L	0.7 (<0.1-3.0)	0.8 (0.2-1.5)	.61
Glucose, mmol/L	4.3 (3.4-5.3)	4.6 (3.6-6.8)	.38
LH, U/L	<0.1 (<0.1-1.4)	<0.1 (<0.1, -0.5)	.92
Estradiol, pmol/L	<70 (<70-< 70)	<70 (<70, -< 70)	>.99
Testosterone, nmol/L	<0.5 (<0.5-< 0.5)	<0.5 (<0.5-< 0.5)	>.99
Triglycerides, mmol/L	1.0 (0.7, 1.8)	0.8 (0.6, 1.7)	.04

Data shown as median (range).
Abbreviation: AMH, anti-Müllerian hormone.
*$P < .05$.

Table 6. Number of arteries incubated per patient for each condition

Case number	No. of arteries per condition			
	Control	E	D	T
1	2	1	2	2
2	1	1	1	1
3	1	1	1	1
4	1	0	1	1
5	1	1	1	1
6	2	2	2	2
7	2	1	1	2
8	1	1	1	1
9	2	2	2	2
10	2	1	1	2
11	2	2	2	2
Control number	**No of arteries per condition**			
	Control	E	D	T
1	1	1	1	1
2	2	2	2	2
3	2	1	1	2
4	3	2	2	2
5	1	1	1	1
6	2	1	1	2
7	2	1	2	1
8	1	1	2	2
9	1	1	1	1
10	1	1	1	1
11	1	1	1	1
12	2	2	2	2

Abbreviations: D, dihydrotestosterone; E, estradiol; T, testosterone.

Androgen and Estrogen Receptor Expression in VSMCs

There was no difference in VSMC expression of *AR* mRNA (Fig. 3A) or protein (Fig. 3C) between boys with hypospadias and controls. VSMCs from boys with hypospadias had increased expression of ESR1 (2.7-fold, $P = .02$), ESR2 (2.6-fold, $P = .04$), and GPER (2.9-fold, $P = .0003$) (Fig. 3B).

Discussion

Interest in the sex differences associated with disease has increased in recent years, with a view to narrowing the gender gap in cardiovascular outcomes. Many of these differences are likely attributable to the effects of testosterone and estrogen. There is a paucity of studies investigating the role of testosterone and estrogen ex vivo in arteries of healthy children. Vascular structure and function change from childhood to adulthood. In particular with aging, there is decreased arterial compliance, increased arterial stiffening, vascular remodelling (28), and reduced endothelial-mediated vasodilation (29). As such, studies like these are important to understand the effects of sex steroids in youth.

Vascular reactivity studies in arteries from rats demonstrated that androgens increase vasorelaxation in normotensive (WKY) and hypertensive (SHR) animals (30). In resistance arteries from human gluteal biopsies from healthy men, supplementation with testosterone reduced the normal dilating response to acetylcholine and sodium nitroprusside but did not affect contractility in response to U46619 (31). In our study, testosterone increased contraction without influencing vasorelaxation in arteries from healthy boys. Similar to testosterone, we found that in control boys, 17β-estradiol increased contraction. Whereas testosterone had no effect on vasodilation in controls, 17β-estradiol reduced vasodilation. Together, these findings suggest that estradiol promotes a contractile phenotype in arteries of young healthy boys. Estrogen has previously been shown to reduce Ca^{2+} influx and act as a vasodilator (32), although some studies suggest that this only occurs at high supraphysiological doses of estrogen (33). Moreover, previous studies that examined the effects of estrogens on vascular function were assessed in vessels from adults. Reasons for these discrepant results between adults and children indicate that vascular reactivity to testosterone changes with ageing. In addition, it is not clear whether the source of the arteries can affect vascular reactivity. Our group previously found no differences in maximal contraction between arteries from subcutaneous penile skin samples or abdominal fat samples although the reactions from penile skin arteries demonstrated increased variability (7). Therefore, although the effects of the steroids differ from those previously reported in adult vessels, it is not clear whether this is because the arteries are from genital skin or from children and future studies should consider this issue in healthy controls.

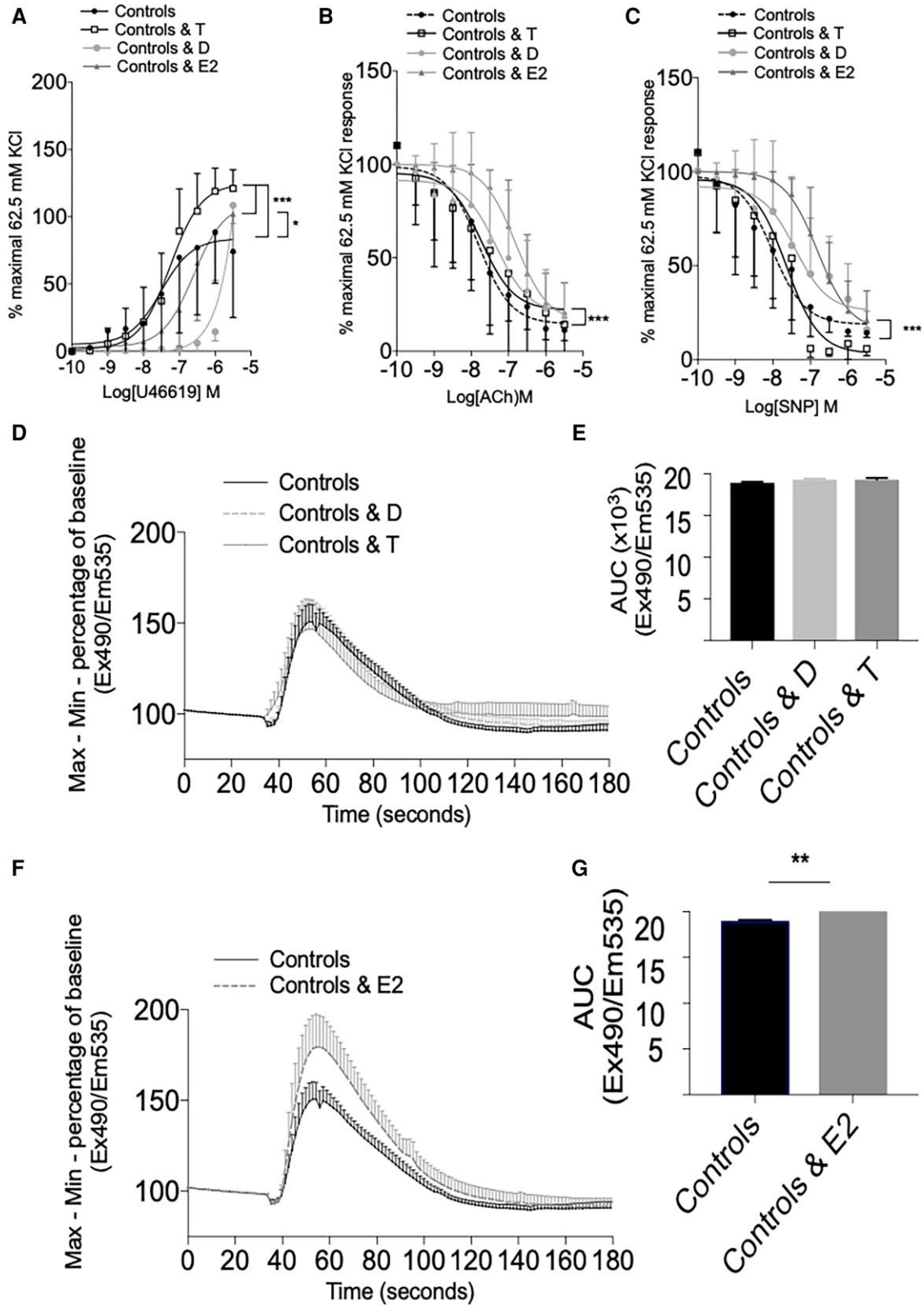

Figure 1. Vascular reactivity and ca2 + influx of resistance arteries from control boys. (A) Arteries from controls demonstrated increased contractility in response to testosterone and 17β-estradiol, with no significant difference in maximal contraction secondary to DHT. (B) Arteries from controls demonstrated no change in response to testosterone or DHT but reduced ACh-dependent vasorelaxation in response to 17β-estradiol. (C) Arteries from controls demonstrated no change in vasorelaxation in response to testosterone or DHT but had reduced vasorelaxation in response to 17β-estradiol. (D, E) Testosterone and DHT did not alter Ca^{2+} influx response to 10^{-6} M U46619 in VSMCs from controls. (F, G) 17β-estradiol increased Ca^{2+} influx in VSMCs from controls (E, F). Results are mean ± SEM of blood vessels from 5 to 9 controls. Where more than 1 blood vessel was obtained from the same boy for the same condition, the mean was used. Best fit cumulative concentration curves were compared with the extra sum-of-squares *F* test. Abbreviations: AUC, area under the curve; D, dihydrotestosterone; E2, 17β-estradiol; KCl, potassium chloride; SEM, standard error of the mean; T, testosterone; V, vehicle. **P* < .05; ****P* < .0001.

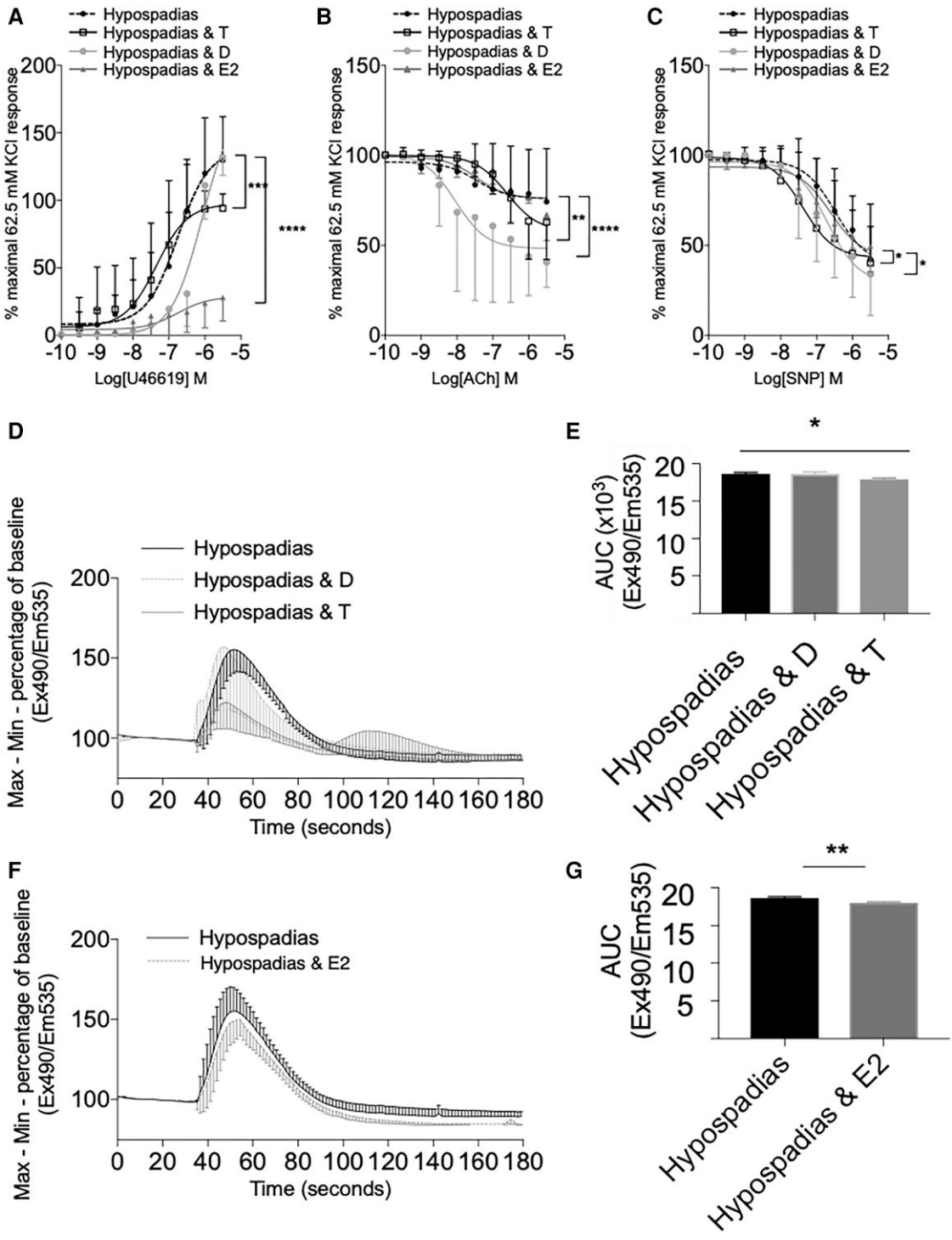

Figure 2. Vascular reactivity and ca2 + influx of resistance arteries from boys with hypospadias. (A) Arteries from boys with hypospadias demonstrated reduced contractility in response to testosterone and 17 β-estradiol, with no significant difference in maximal contraction in response to DHT. (B) Arteries from boys with hypospadias demonstrated increased ACh-dependent vasorelaxation in response to testosterone and DHT but no response to 17β-estradiol. (C) Arteries from boys with hypospadias demonstrated increased vasorelaxation in response to testosterone and DHT but no change in response to 17β-estradiol. (D, E)Testosterone reduced Ca^{2+} influx in VSMCs from boys with hypospadias. DHT had no effect on Ca2+ influx. (F, G) 17β-estradiol reduced Ca^{2+} influx in VSMCs from boys with hypospadias. Results are mean ± SEM of blood vessels from 5 to 9 boys with hypospadias. Where more than 1 blood vessel was obtained from the same boy for the same condition, the mean was used. Best fit cumulative concentration curves were compared with the extra sum-of-squares *F* test. Abbreviations: AUC, area under curve; D, dihydrotestosterone; E2, 17β-estradiol; KCl: potassium chloride; SEM, standard error of the mean; T, testosterone; V: vehicle. $*P < 0.05$; $***P < 0.0001$.

Previously, we have shown that baseline vascular reactivity differs in the arteries from healthy controls and those born with hypospadias with evidence of hypercontractility and impaired endothelium-dependent and -independent vasorelaxation (7). As such, it is suspected that the arteries from boys with hypospadias do not function in the same way as arteries from control boys. In the current study, compared with healthy controls, incubation of testosterone and 17β-estradiol in arteries from boys

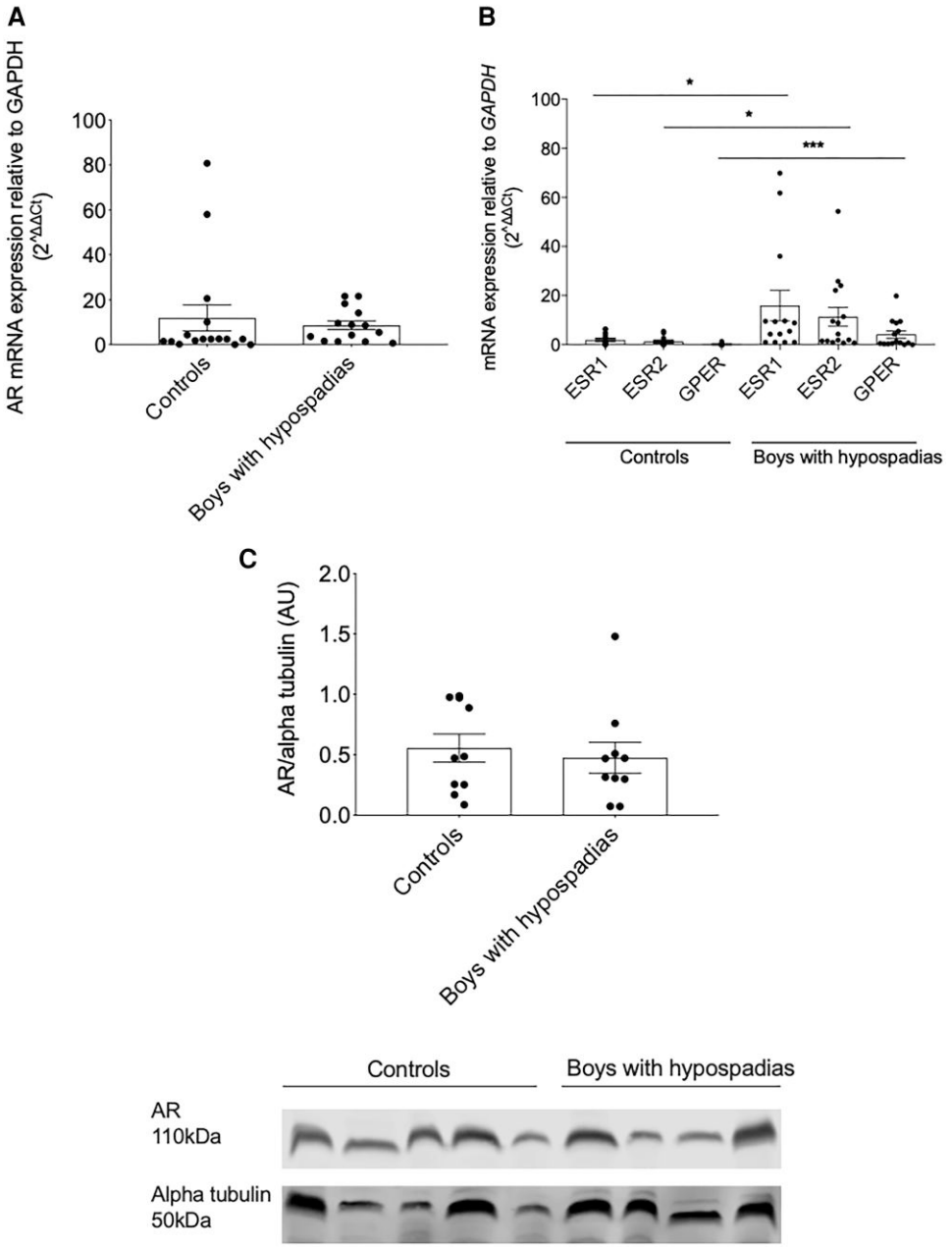

Figure 3. Androgen and estrogen receptor expression in VSMCs from boys with hypospadias and controls. (A) —There were no differences in mRNA expression of the androgen receptor in VSMCS from boys with hypospadias and controls. Results are mean ± SEM from 12 controls and 11 cases. Data were analyzed using Mann-Whitney *U*. (B) ESR1, ESR2, and GPER mRNA expression were upregulated in VSMCS from cases compared with controls. Results are mean ± standard error of the mean (SEM) from 12 controls and 11 cases. Data were analyzed using 1-way ANOVA followed by Tukey posttest correction. (C) There were no differences in protein expression of the androgen receptor in VSMCs from boys with hypospadias and controls. Results are mean ± SEM from 10 controls and 10 cases. Data were analyzed using Mann-Whitney *U*. $*P < 0.05$, $***P < 0.001$.

with hypospadias reduced the hypercontractility induced by U46619, with estrogen inhibiting contraction almost completely. In comparison, 17β-estradiol increased contractility in arteries from control boys. These findings suggest that vessels from boys with hypospadias lose their responsiveness to estrogens. Testosterone increased endothelium-dependent and -independent vasodilation but 17β-estradiol had no effect on vasodilation, indicating that vasorelaxation is more likely to be a direct effect of androgens. This is consistent with studies reporting increased vasorelaxation in response to androgens independent of aromatization in vessels from spontaneously hypertensive rats (34). Hypogonadism is associated with increased oxidative stress, a phenomenon that has also been described in children with hypospadias (7). Other mechanistic studies have shown that testosterone improves vascular reactivity in orchidectomized rats via improvement of the antioxidant milieu (35). In VSMCs from cases, testosterone reduced Ca^{2+} influx, in line with reduced contraction whereas in controls, vascular contraction was increased in response to testosterone but with no apparent alteration in Ca^{2+} influx. 17β-estradiol also reduced Ca^{2+} influx in cases, which is consistent with the finding of potential ESR unresponsiveness.

DHT had no effect on contractility in arteries from cases but increased endothelium-dependent and -independent vasodilation. Local application of 2.5% DHT gel is a practice used by some pediatric urologists before hypospadias repair to increase penile length (36). However, this clinical approach remains variable, in part because of data suggesting that androgen therapy in this way may increase operative complication rate (37, 38). The role of DHT in the vasculature merits further investigation, in particular because of its different actions compared with testosterone in these arteries.

Molecular mechanisms contributing to differences in vascular reactivity to sex hormones between children and adults in health and disease remain unclear but may relate, in part, to differential expression of sex hormone receptors and aromatase during aging and with disease. Androgen receptor and aromatase expression differ according to the vascular site in men with coronary artery disease, although estrogen receptor expression remains constant (39). In preputial tissue of boys with hypospadias, expression of estrogen receptors (ESR1 and ESR2) has been reported to be reduced (40). However, in our study, we found an increase in mRNA expression of ESR1, ESR2, and GPER. Polymorphisms in ESR1 and ESR2 are reported to be associated with hypospadias, with variants in ESR1 particularly thought to alter function of ER-α protein (41). In addition, polymorphisms in ESR1 are associated with increased risk of stroke (42), endothelial dysfunction and vascular risk (43), and preeclampsia (44). We also found impaired VSMC Ca^{2+} influx in boys with hypospadias. Because changes in intracellular free Ca^{2+} levels are critically involved in regulating contraction/dilation, the alterations we observe may contribute to impaired vascular function in hypospadias.

Hypospadias is associated with a period of apparent hypogonadism during fetal development. Endocrine disruptor chemicals with estrogenic or antiandrogenic properties have been implicated in the pathophysiology of hypospadias (45). Hypospadias can also be induced experimentally by exposures that interfere with androgen and estrogen synthesis or action (46). Estrogen receptor gene disruption and estrogen can also cause severe congenital penile anomalies (47). The differences in sex steroid signalling in boys with hypospadias and controls leads to the question regarding why cases and controls would have discrepant responses to testosterone and estrogen. This may reflect the consequences of the underlying etiology of hypospadias and should be the focus of future studies, for example investigating particular groups of boys with hypospadias, such as those with androgen receptor or other genetic variants.

The key limitation of this study is the relatively low number of participants. Although the confidence intervals in most of the studies suggest the results are likely to be reproducible, a larger cohort would improve the validity of results, particularly those in which previous studies in different species or settings have demonstrated opposite results. It is possible that the effects seen in boys with hypospadias are limited by the impaired vascular reactivity already present, for example, with the arteries that have reached the limit of hypercontractility and therefore being unable to constrict any further. In this circumstance, however, it would not be expected that the sex steroids would significantly reduce vasoconstriction, as we have seen in the presence of testosterone and 17β-estradiol, but rather would remain maximally constricted.

In conclusion, we demonstrate that androgens and estrogens have contrasting effects on vascular reactivity in arteries of young boys, and these effects are different in those with hypospadias. In healthy boys, testosterone and 17β-estradiol promote a vasoconstrictor phenotype, whereas in boys with hypospadias, these sex hormones reduce vasoconstriction, with testosterone and DHT promoting vasorelaxation. Reasons for these functional differences are unclear but may relate to altered sex hormone receptor status, particularly ESR1, ESR2, and GPER in hypospadias. The long-term vascular impact of alterations in early life exposure to sex hormones requires further study.

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Disclosures

There are no conflicts to declare.

Data Availability Statement

Data are available on reasonable request.

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