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Original Research

Long-term effects of vaginal surgery and endogenous ovarian hormones on the vagina and bladder

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Abstract

Background: Surgery is a common treatment for pelvic organ prolapse (POP); however, risk of recurrence and reoperation is high, resulting in a negative impact on quality of life and sexual function.
Aim: To examine the long-term effects of POP surgery and endogenous circulating ovarian hormones on the vagina and bladder.
Methods: Our animal model simulated surgical injury of the vagina and bladder during POP surgery. Female Rowett nude rats were divided into 4 groups: intact control (IC), vaginal surgery only (V), ovariectomy only (O), and ovariectomy + vaginal surgery (OV). Rats were euthanized 10 weeks postsurgery. Proximal vagina and bladder dome/trigone underwent (1) organ bath myography to assess smooth muscle contractility; (2) real-time quantitative polymerase chain reaction to quantify mRNA expression of elastin, collagen I and III, and PGP9.5 (protein gene product 9.5); (3) enzyme-linked immunosorbent assay for protein quantification of elastin and collagen I and III; and (4) hematoxylin-eosin/immunohistochemistry staining.
Outcomes: The primary outcome was tissue contractility as measured by organ bath myography. Secondary outcomes included gene and protein expression of collagen I and III and elastin.
Results: O and OV showed reduced vaginal wall contractility vs IC and V ($P < .002$). Bladder dome and trigone displayed different contractile patterns, with significant differences between O and OV ($P < .05$), suggesting a negative effect from surgery rather than ovariectomy. OV demonstrated consistent reductions in contractility and elastin/collagen protein expression for the vagina and bladder vs IC. V had similar contractility and increased collagen I expression vs IC, suggesting a protective effect of ovarian hormones. Vaginal epithelium thinning was confirmed in the ovariectomized groups ($P = .001$), although there was no statistical significance in muscularis thinning with surgery or ovariectomy. O, V, and OV showed significant downregulation of PGP9.5 mRNA expression vs IC.
Clinical Translation: These data allow researchers to gain insights into the long-term effects of surgery and deprivation of ovarian hormones. Future studies can use this animal model to investigate other mechanisms that may affect long-term tissue changes due to surgical intervention.
Strengths and Limitations: Major strengths are long-term data on the effects of POP surgery and development of an animal model for future studies. However, the animal model limits our ability to extrapolate to humans, where tissue healing is modulated by many factors.
Conclusion: Our animal model provides evidence that ovarian hormone deprivation and POP surgery result in negative long-term effects on tissue function and extracellular matrix.

Keywords: prolapse vaginal surgery; endogenous ovarian hormones; organ bath myography; extracellular matrix; vagina; vaginal contractility; bladder contractility; rat model of prolapse surgery.

Introduction

Sexual dysfunction is associated with pelvic organ prolapse (POP).[1,2] POP is a prevalent and chronic condition characterized by the descent and protrusion of the vagina or ≥1 pelvic organs from the vaginal introitus. Significant risk factors include parity and age.[3] Symptoms vary from mild discomfort to severe pain, and it is associated with urinary incontinence and defecation problems.[4] POP can also result in reduced genital sensation and negative effects on sexual function. While treatment options including physiotherapy and pessary use, many patients ultimately opt for surgery. The lifetime risk for an 80-year-old woman undergoing surgery for POP is an estimated 13% to 19% in different continents.[5-7] After surgery, average rates of recurrence and reoperation are roughly 25%-30% and 17%, respectively.[8,9] Therefore, many women will undergo POP surgery as they age.

Vaginal surgery with native tissue repair, instead of synthetic mesh implants to augment tissue properties, is frequently performed to avoid mesh-associated risks, such as chronic dyspareunia and pelvic pain. While most studies reveal an improvement in sexual function after vaginal surgery with native tissue repair, a meta-analysis evaluating the effect of vaginal surgery with native tissue on sexual function reported that roughly two-thirds of the women stated that their dyspareunia was unchanged or had worsened (39% and 18%).[10] The common dogma on postoperative de novo dyspareunia is that it is due to surgical scarring of the vagina and surrounding structures. However, little is known about the underlying long-term tissue changes resulting from surgery that may lead to negative effects on sexual function and POP recurrence.

The question that we ask in this study is as follows: Are there long-term changes with respect to tissue contractility,

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extracellular matrix (ECM) proteins, and a neuronal marker in the vaginal wall and adjacent bladder due to POP vaginal surgery? We used a rat model to investigate this. Since women who undergo POP surgery may be pre- or postmenopausal, we also studied the effect of endogenous ovarian hormones associated with POP surgery. We hypothesize that the combination of vaginal surgery and hormone deprivation is necessary for long-term negative tissue effects in the vagina and bladder. There is limited knowledge on the effect of ovarian hormones on long-term vaginal surgery healing.[11] Findings from our study could help elucidate the underlying mechanisms that contribute to altered sexual function after vaginal surgery for prolapse.

Methods

Animal groups and surgery

We used female immunodeficient Rowett nude rats (Charles River Laboratories; age, 63-83 days; weight, 170-200 g). This rat was selected because our future studies will involve testing of human stem cell–derived progenitor cells in this animal model. Animal protocols were approved by the Institutional Review Board of the Stanford University School of Medicine and Stanford Administrative Panel of Laboratory Animal Care (33817/IRB 9992).

Surgical procedures

Rats were anesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg). An abdominal midline incision was made, followed by ligation of the adnexal vascular supply and resection of both ovaries. For vaginal surgery, the bladder was retracted and urethrolysis performed to separate the urethra and bladder trigone from the anterior vagina, as described by Rodríguez et al.[12] Once the anterior vagina was exposed, 2 cuts were made in the anterior vaginal muscularis layer. This area was then clamped with a hemostat for 5 minutes. The bladder was placed in its normal position and the abdominal incision closed.

Study design

Rats were divided into the following groups:

1) IC: intact control (n = 15)
2) O: ovariectomy only (n = 10)
3) OV: ovariectomy and vaginal surgery (n = 31)
4) V: vaginal surgery only (n = 14)

Our preliminary data showed that an extensive recovery time was necessary for resolution of acute inflammatory changes and the smooth muscle hypersensitivity observed by Callewaert et al at 6 weeks after vaginal injury.[13] Therefore, rats were euthanized and tissue harvested 10 weeks postsurgery. Proximal vagina tissues were used for organ bath myography and protein expression analysis. Rats in all surgical groups were injected with 200 μL of sterile saline into lateral sides of the anterior vaginal wall (100 μL/side) 5 weeks after surgery. This was done so that these groups could be used as sham controls for data in future studies.

Organ bath myography

Tissues were weighed and measured after harvest. Optimal resting tension was determined through preliminary studies (vagina, 1.03 g; bladder dome, 1.08 g; bladder trigone, 1.03 g). Proximal vagina tissues were mounted circumferentially,[14,15] whereas bladder dome and trigone tissues were mounted longitudinally. The strips were immersed in 25-mL organ baths oxygenated with 95% O_2 and 5% CO_2 at 37 °C in Kreb buffer (118mM NaCl, 2.5mM $CaCl_2$, 4.7mM potassium chloride [KCl], 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 11mM glucose, 25mM $NaHCO_3$, pH 7.4). The strips were then set to their corresponding optimal resting tension and allowed to equilibrate for 60 minutes. Contractile responses were monitored with a custom-made isometric force transducer, and signals were recorded with Lab Chart 7 (AD Instrument).

We assessed muscle-mediated contraction using KCl solution: 40mM KCl for vagina and 160mM KCl for bladder tissues. Once contractile response plateaued, tissues were washed 3 times with Krebs buffer. Carbachol—a nonselective muscarinic receptor agonist (Sigma-Aldrich) in cumulative concentrations of 0.625, 1.25, 2.5, 5, 10, and 20 μM—was used to assess receptor function. Strips were washed again before verifying true carbachol-stimulated response by adding 1μM atropine, an anticholinergic drug, for 5 minutes and stimulating strips again with carbachol (20 μM; Figure S1). Tissues were washed and viability checked with initial KCl concentrations. Tissue contractility data were normalized to the tissue area and expressed as tension per unit of tissue area (grams per square centimeter).

RNA extraction and reverse transcription

RNA from tissues was extracted via homogenization in RNA-STAT-60 reagent (Tel-Test) as described previously.[16] RNA yield was determined with a Nanodrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription was done with the M-MLV reverse transcriptase system[16] (Thermo Scientific).

Real-time quantitative polymerase chain reaction was used to evaluate mRNA expression of elastin, collagen I and III, smooth muscle markers (α-smooth muscle actin [SMA], smoothelin [SMT]), and a general neuronal marker (protein gene product 9.5 [PGP9.5]). Primer sequences are shown in the supplemental documents. Real-time quantitative polymerase chain reaction was performed in duplicates on G8830A Aria Mx Real-time PCR with Brilliant SYBR Green qPCR Master Mix (Agilent Technologies). The thermal profile was set to 95 °C (10 minutes; ie, hot start) at 40 cycles of amplification, followed by 95 °C (30 seconds), 55 or 60 °C (1 minute), and 72 °C (1 minute) and concluding with 1 cycle of 95 °C (1 minute), 55 °C (30 seconds), and 95 °C (30 seconds). The Ct method (cycle of threshold) was used for quantification. Relative gene quantification—corrected for the quantity of the normalizer gene (GAPDH)—was divided by 1 normalized control sample value (calibrator sample) to generate the relative quantification to the calibrator. The calculations were done by Aria Mx 2.0 software (Agilent Technology). The primers' sequences are listed in Supplementary Table 1.

Histochemical and immunohistochemical assays

We deparaffinized tissue slides in xylene and sequentially hydrated them using 100%, 95%, and 70% deionized water. Tissue slides were then stained with hematoxylin solution (Mayer's; Abcam), rinsed, and counterstained with eosin Y (0.5% alcoholic solution; Polysciences).

For immunohistochemistry staining against PGP9.5, deparaffinized slides were rinsed in Tris- buffered saline–0.2% Tween (TBS-T) before endogenous peroxidase was blocked

with 3% hydrogen peroxide for 15 minutes, followed by an antigen retrieval step consisting of 4 cycles of boiling in 0.1M citrate buffer (pH 6.0) for 2.5 minutes per cycle. Slides were incubated for 1 hour in a blocking buffer—5% goat serum (Sigma) in TBS-T with 1% bovine serum albumin—and then incubated at 4 °C overnight with rabbit anti-PGP9.5 (2 μg/mL; Abcam) or rabbit immunoglobulin G (1 μg/mL; Sigma) for negative slides in blocking buffer. Sections were rinsed in TBS-T before secondary incubation in biotinylated goat anti-rabbit, (1:50; Sigma) for 1 hour at room temperature. The sections were washed in TBS-T and incubated in a mixture of avidin-biotinylated alkaline phosphatase diluted in TBS-T (Vectastain ABC-AP Kit; Vector Laboratories) for 30 minutes at room temperature. After wash, the slides were incubated in Vector Red reagent (Vector Red Alkaline Phosphatase Substrate Kit) diluted in 0.1M Tris-HCl (pH 8.3) with 1 drop of Levamisole solution (Vector Laboratories) for 30 minutes at room temperature in the dark. Slides were rinsed and counterstained with hematoxylin solution (Mayer's; Abcam).

Image quantification

Zen software (blue edition, version 3.4; Zeiss) was used to quantify tissue wall thickness from hematoxylin and eosin slides. Six measurements of the tissue wall were recorded: the thickness of the lamina propria and muscularis layers of the anterior vagina and epithelium.

Enzyme-linked immunosorbent assay

Protein expression of elastin and collagen I and III was quantified in duplicate with enzyme-linked immunosorbent assay kits (Lifespan Biosciences) per the manufacturer's instruction. Optical absorbance was measured with a spectrophotometer (SpectraMax M3; Molecular Devices). Quantification of target proteins was calculated by its standard curve and then normalized to the concentrations of the protein (milligrams per milliliter) in the samples.

Data analysis

The nonparametric Wilcoxon test was applied for statistical comparisons of groups with JMP Pro 16 software (SAS Institute). Statistical significance was set at $P < .05$.

Results

Tissue contractile function

Vagina

We observed significant reductions in vaginal contractility in response to KCl stimulation in ovariectomized groups with (OV) or without (O) vaginal surgery as compared with IC ($P = .0001$ and .0013, respectively). Similarly, we saw significant reductions when comparing the ovariectomized groups (O, OV) with the V group ($P < .0012$; Figure 1). There was no difference in contractility between the IC and V groups.

For stimulation with carbachol (a muscarinic receptor agonist), ovariectomized groups (O, OV) revealed decreased responses throughout stimulation with increasing carbachol concentrations as compared with the nonovariectomized groups (IC, V; Figure 2A). At high carbachol concentrations (10 and 20 μM; Figure 2B), the significant differences among the groups are consistent with the differences observed with KCl stimulation (Figure 1). Again, no significant difference in carbachol-stimulation contractility was observed between the nonovariectomized groups (IC, V).

Bladder dome

Normalized contractile responses to KCl stimulation in the bladder dome were similar for all surgical groups when compared with IC. The only significant difference was between O and OV ($P = .016$; Figure 1), with a significant decline in contractility in OV. Unlike the vagina, O and V showed similar response curves to carbachol and were higher than IC and OV, with OV showing the lowest dose-response curve (Figure 2A). At 20 μM, carbachol-stimulated contractions in OV were significantly weaker than O and V ($P = .003$ and .03, respectively; Figure 2B). There were no significant differences between any of the surgical groups and IC at 20 μM (Supplementary Figure 2).

Bladder trigone

The bladder trigone showed significantly stronger KCl-stimulated contractions in the V group as compared with all other groups (IC, O, and OV; $P = .02$, .02, and .01). Additionally, OV group contractions were significantly lower than those of the O group ($P = .03$; Figure 1).

Carbachol stimulation responses for the bladder trigone exhibited the same pattern as KCl-stimulated contractions (Figure 2A). At 20 μM, V yielded stronger contractions than IC, O, and OV ($P = .0003$, .01, and .0002; Figure 2B, supplementary Figure 3).

Gene expression

We evaluated mRNA expression of ECM proteins (elastin, collagen I, collagen III), smooth muscle cell markers (SMA and SMT), and a peripheral nerve marker (PGP9.5) in the vagina and bladder tissues. For the vagina, O and OV showed trends toward downregulation of mRNA expression of elastin and collagen I and III as compared with IC, while V showed a trend toward upregulation of these proteins vs IC. PGP9.5 was downregulated in all groups (O, OV, and V) vs IC (Table 1). Trigone and dome tissues revealed downregulation of elastin, collagen I and III, and PGP9.5 mRNA expression in O and OV as compared with IC.

We tested a subgroup of vaginal tissues that had not undergone organ bath myography prior to polymerase chain reaction analysis, in case the organ bath may have affected the results. This subgroup analysis showed no significant difference in elastin, SMA, and SMT mRNA expression between IC and OV but confirmed a significant decrease in collagen I and III mRNA expression in OV ($P < .001$; Table 2).

Protein expression

Histologic stains for elastin and collagen of the vagina and bladder tissues illustrate qualitative changes in elastin between IC and the other groups (Figure 3). Elastin fibers were long and in parallel formation in IC, as opposed to disorganized and difficult to locate in O and OV. Because changes in ECM are heterogenous, it is difficult to reliably quantify changes by histomorphologic analysis. We therefore relied on gene and protein quantification to demonstrate differences among the groups.

Vagina

Elastin protein expression was significantly higher in IC and O as compared with OV ($P = .003$). V also had significantly

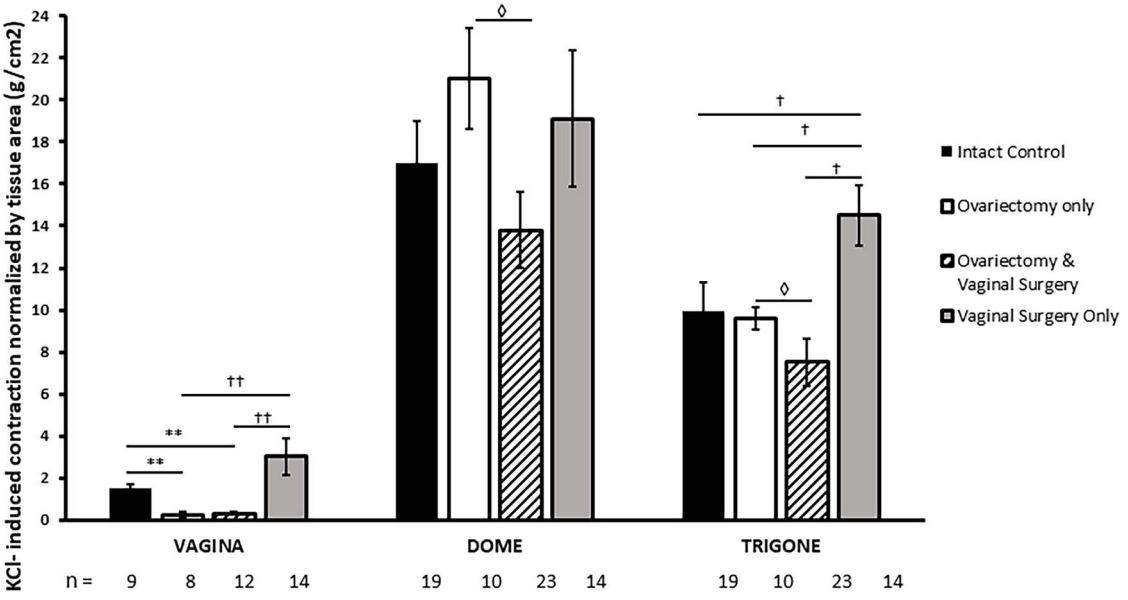

Figure 1. Tissue contraction in response to KCl. Data are normalized to tissue area. Vagina was subjected to 40mM KCl. Bladder (dome and trigone) was subjected to 160mM KCl. Error bars indicate SEM. Sample size is included below each column. $^{*}P < .05$. $^{**}P < .01$ vs intact control. $^{\dagger}P < .05$. $^{\dagger\dagger}P < .01$ vs ovariectomy + vaginal surgery. $^{\Diamond}P < .05$. $^{\Diamond\Diamond}P < .01$ vs ovariectomy only. KCl, potassium chloride.

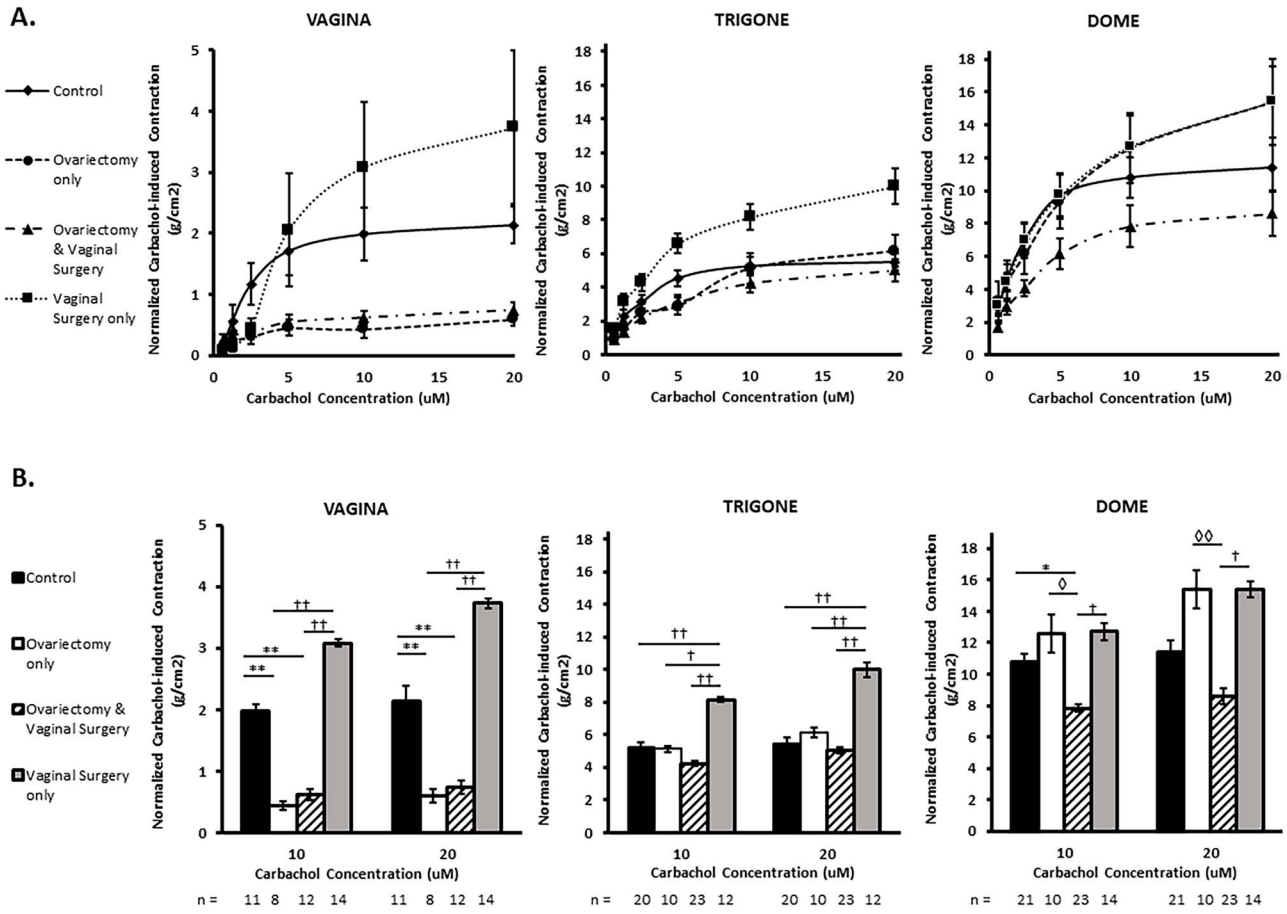

Figure 2. (A) Carbachol response curve: vagina, bladder dome, and bladder trigone. Carbachol concentrations: 0.625, 1.25, 2.5, 5, 10, and 20 μM. Vagina: Measured contractions are normalized to tissue area. Intact control (n = 11), ovariectomy only (n = 8), ovariectomy + vaginal surgery (n = 12), vaginal surgery only (n = 14). Bladder trigone: intact control (n = 20), ovariectomy only (n = 10), ovariectomy + vaginal surgery (n = 23), vaginal surgery-only (n = 12). Bladder dome: Intact control (n = 21), ovariectomy only (n = 10), ovariectomy + vaginal surgery (n = 23), (B) Mean contraction at 10 and 20 μM. Error bars measure SEM. Sample size is included below each column. Error bars measure SEM. $^{*}P < .05$. $^{**}P < .01$ vs intact control. $^{\dagger}P < .05$. $^{\dagger\dagger}P < .01$ vs ovariectomy + vaginal surgery. $^{\Diamond}P < .05$. $^{\Diamond\Diamond}P < .01$ vs ovariectomy only.

Table 1. RT-qPCR gene expression quantification of extracellular matrix markers and nerve marker in the vagina and bladder of the surgical groups vs intact control group.[a]

Organ: marker	Ovariectomy only		Ovariectomy + vaginal surgery		Vaginal surgery only	
Vagina						
Elastin						
Control	1.80 ± 0.505 (n = 13)	↓	0.333 ± 0.074 (n = 12)	↓	0.149 ± 0.035 (n = 8)	↑
Surgery	1.37 ± 0.192 (n = 9)		0.244 ± 0.036 (n = 10)		0.281 ± 0.055 (n = 12)	
P value	.815		.553		.076	
Collagen I						
Control	0.544 ± 0.116 (n = 13)	↓	3.35 ± 0.819 (n = 13)	↓	1.58 ± 0.581 (n = 8)	↑
Surgery	0.285 ± 0.112 (n = 9)		2.88 ± 0.523 (n = 11)		3.78 ± 0.512 (n = 12)	
P value	.057		.885		**.009**	
Collagen III						
Control	1.44 ± 0.237 (n = 13)	↓	7.70 ± 1.44 (n = 12)	↓	4.02 ± 0.815 (n = 9)	↑
Surgery	0.982 ± 0.162 (n = 9)		6.70 ± 1.49 (n = 10)		6.91 ± 0.141 (n = 13)	
P value	.193		.510		.367	
PGP9.5						
Control	27.6 ± 20.9 (n = 13)	↓	5.84 ± 3.51 (n = 7)		18.8 ± 8.79 (n = 8)	↓
Surgery	2.54 ± 1.81 (n = 5)		3.79 ± 1.29 (n = 10)		1.07 ± 0.291 (n = 7)	
P value	.349		.922		.105	
Dome						
Elastin						
Control	5.87 ± 0.672 (n = 13)	↓	3.14 ± 0.415 (n = 13)	↓	0.835 ± 0.116 (n = 9)	↓
Surgery	2.56 ± 0.505 (n = 9)		0.631 ± 0.073 (n = 11)		0.719 ± 0.106 (n = 13)	
P value	**.004**		**<.0001**		.483	
Collagen I						
Control	7.51 ± 1.38 (n = 13)	↓	3.19 ± 0.447 (n = 11)	↓	4.74 ± 0.782 (n = 9)	↓
Surgery	1.55 ± 0.563 (n = 9)		0.642 ± 0.200 (n = 10)		2.30 ± 0.462 (n = 13)	
P value	**.0007**		**.0003**		**.010**	
Collagen III						
Control	17.2 ± 2.53 (n = 13)	↓	12.1 ± 1.25 (n = 13)	↓	13.8 ± 1.46 (n = 9)	↓
Surgery	8.48 ± 2.87 (n = 9)		7.28 ± 2.18 (n = 11)		10.3 ± 1.58 (n = 13)	
P value	**.042**		**.022**		.243	
PGP9.5						
Control	3.24 ± 0.562 (n = 13)	↓	9.54 ± 2.21 (n = 11)	↓	1.12 ± 0.197 (n = 11)	
Surgery	1.52 ± 0.428 (n = 9)		3.48 ± 1.32 (n = 9)		0.787 ± 0.188 (n = 11)	
P value	**.049**		**.007**		.140	
Trigone						
Elastin						
Control	5.87 ± 0.672 (n = 13)	↓	0.967 ± 0.122 (n = 13)	↓	0.684 ± 0.108 (n = 10)	
Surgery	2.56 ± 0.513 (n = 9)		0.280 ± 0.058 (n = 10)		0.62 ± 0.105 (n = 14)	
P value	**.004**		**.0003**		.640	
Collagen I						
Control	3.41 ± 0.632 (n = 13)	↓	3.16 ± 0.797 (n = 12)	↓	2.70 ± 0.688 (n = 9)	↑
Surgery	1.20 ± 0.307 (n = 9)		0.658 ± 0.145 (n = 10)		4.63 ± 0.899 (n = 13)	
P value	**.018**		**.003**		.151	
Collagen III						
Control	5.98 ± 0.756 (n = 13)	↑	17.2 ± 4.18 (n = 12)	↓	16.2 ± 4.07 (n = 9)	↑
Surgery	13.0 ± 3.74 (n = 9)		5.38 ± 1.26 (n = 10)		20.1 ± 4.02 (n = 13)	
P value	.301		**.025**		.617	
PGP9.5						
Control	6.12 ± 0.974 (n = 13)	↓	12.1 ± 2.04 (n = 11)	↓	2.40 ± 0.493 (n = 10)	
Surgery	2.54 ± 0.383 (n = 9)		1.51 ± 0.379 (n = 10)		1.30 ± 0.196 (n = 11)	
P value	**.018**		**.0004**		.105	

Abbreviations: PGP9.5, protein gene product 9.5; RT-qPCR, real-time quantitative polymerase chain reaction. [a]RT-qPCR quantification of gene expression of extracellular matrix markers (elastin, collagen I, collagen III) and nerve marker (PGP9.5) in the vagina and bladder of the ovariectomy-only, ovariectomy + vaginal surgery, and vaginal surgery–only groups as compared with the intact control group. Data are expressed as mean ± SEM. Bold *P* values indicate significant difference vs intact controls. Arrows indicate direction of gene fold change vs intact controls. RT-qPCR was performed with post–organ bath tissues.

higher elastin expression than OV (P = .03). There was no difference in elastin expression between IC and V (Figure 4).

Collagen I protein expression was higher in the V as compared with IC, O, and OV (P = .0004). We also observed statistical differences in IC vs O and OV (P = .001). For collagen III, we saw elevated protein expression in V vs O and OV (P = .01).

Bladder dome

Elastin expression in the bladder dome was similar between IC and the ovariectomy groups. However, surgery with and without ovariectomy resulted in a significant decrease in dome elastin expression (OV, P = .034; V, P = .010).

Collagen I was most abundant in the IC group, significantly higher than all remaining groups: O (P = .013), OV (P = .001), and V (P = .0004). In contrast, collagen III was lowest in the V group as compared with every other group: IC (P = .005), O (P = .027), and OV (P = .034; Figure 4).

Bladder trigone

In the bladder trigone, we observed a significant decrease in elastin in OV vs IC (P = .004), as well as between O and

Table 2. mRNA expression in the vaginal tissues of the ovariectomy + vaginal surgery vs intact control groups.[a]

Organ: marker	RNA expression			
	Intact control (n = 5)	Ovariectomy + vaginal surgery (n = 16)	*P* value	Direction of change vs intact control
Vagina				
Elastin	2.04 ± 0.606	2.12 ± 0.683	.553	
Collagen I	4.74 ± 1.31	0.68 ± 0.164	**.0013**	↓
Collagen III	7.11 ± 0.880	1.32 ± 0.265	**.001**	↓
SMA	1.62 ± 0.229	1.92 ± 0.304	.934	
SMT	5.16 ± 1.48	6.08 ± 1.85	.55	

Abbreviations: RT-qPCR, real-time quantitative polymerase chain reaction; SMA, α-smooth muscle actin; SMT, smoothelin. [a]mRNA expression of elastin, collagen I, collagen III, SMA, and SMT in the vaginal tissues of the ovariectomy + vaginal surgery group as compared with the intact control group. Arrow indicates direction of gene fold change vs intact controls. Data are expressed as mean ± SEM. Bold *P* values indicate significant difference. RT-qPCR was performed with fresh (non–organ bath) tissues.

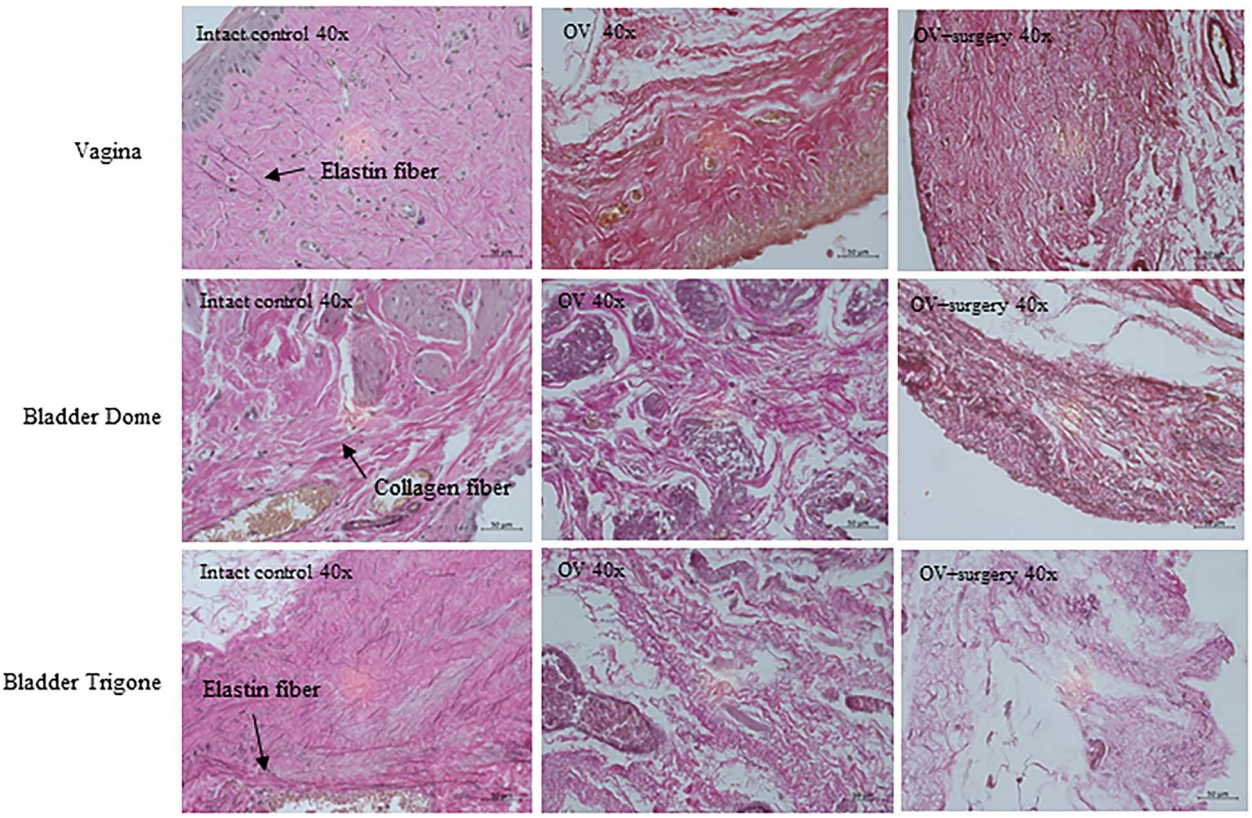

Figure 3. Morphology of collagen and elastin fibers in the bladder and vaginal wall. Sections were stained with van Gieson (collagen) and Weigert resorcin-fuchsin (elastin). Scale bars: 50 μM. Elastin stain showed that elastin fibers were longer and more organized in the intact control group. No difference was observed between the ovariectomy and ovariectomy + vaginal surgery groups. OV, ovariectomy.

OV(*P* = .002; Figure 4). There was no difference between IC and V.

There were no significant differences in collagen I expression in the trigone among the groups. However, significant differences were seen in collagen III expression. OV and V showed lower collagen III expression as compared with IC (*P* = .034 and 0.011, respectively). Ovariectomy increased collagen III expression when compared with IC. Finally, OV was more abundant in collagen III than V (*P* = .005).

Vaginal wall thickness

There was no statistically significant difference in the thickness of the muscularis and lamina propria between IC and OV from proximal and middle segments of the vagina. However, there was a trend toward a thinner muscularis layer in OV as compared with IC (Figure 5).

Epithelium of the vagina was significantly thicker in IC than OV (*P* = .001). Serum testosterone levels in rats 10 weeks postovariectomy were below the detection level of the assay (Saint Louis Zoo; Endocrinology Laboratory).

Neuronal marker

We performed immunohistochemical stains to examine localization of the PGP9.5 protein in the vagina and bladder tissues. Overall, there was scant PGP9.5 staining throughout the different layers of the vagina. PGP9.5 was localized in the vaginal and bladder lumen, along the epithelial layer (data not shown). Nerve bundles were observed in the lamina propria

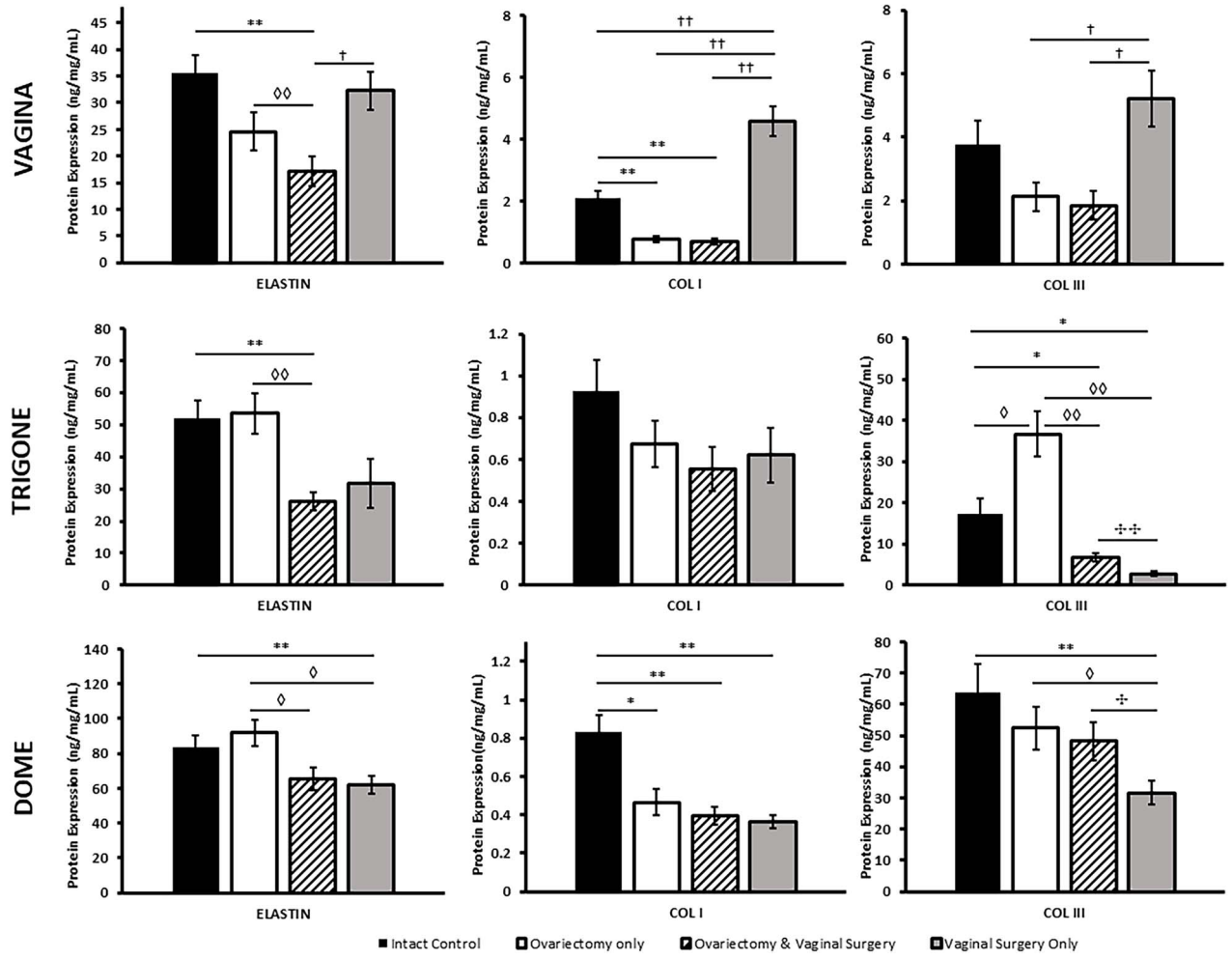

Figure 4. Protein expression of elastin and collagen I and III in the vagina and bladder. Protein expression normalized to protein concentration in sample. Error bars measure SEM. $^{*}P < .05$. $^{**}P < .01$ vs intact control. $^{\dagger}P < .05$. $^{\dagger\dagger}P < .01$ vs ovariectomy + vaginal surgery. $^{\Diamond}P < .05$. $^{\Diamond\Diamond}P < .01$ vs ovariectomy only. $^{\ddagger}P < .05$. $^{\ddagger\ddagger}P < .01$ vs vaginal surgery only.

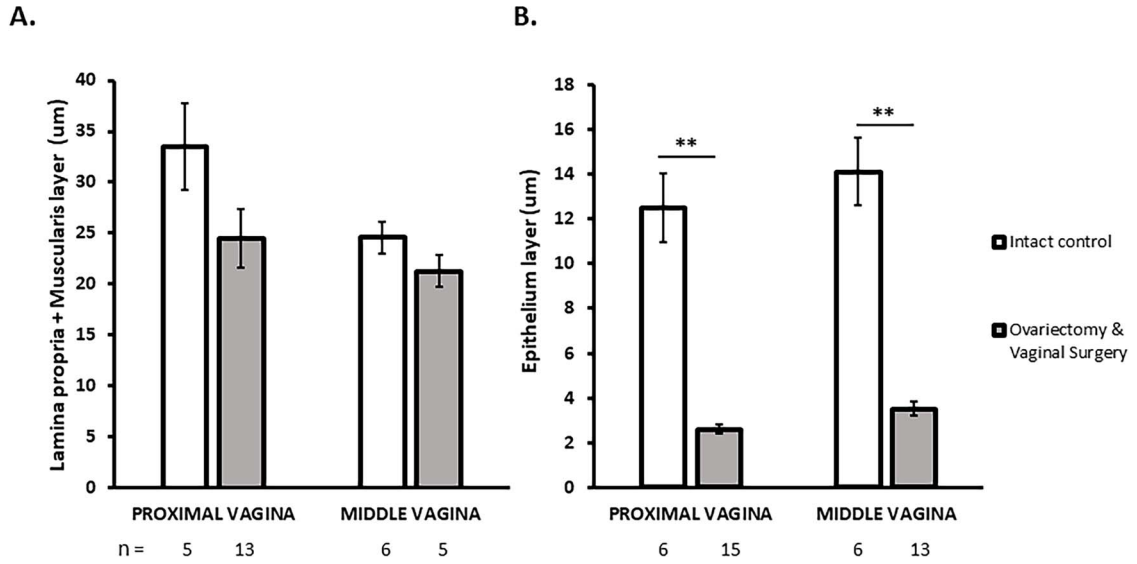

Figure 5. (A) Quantification of lamina propria and muscularis thickness in proximal and middle vagina wall. (B) Quantification of epithelium layer in proximal and middle vagina. Sample size is included below each column. Error bars indicate SEM. $^{*}P < .05$. $^{**}P < .01$.

of the vagina. There was a visible decrease in PGP9.5 staining in the vagina and bladder tissues in OV as compared with IC, reflective of the decrease in gene expression data.

Discussion

This study aimed to investigate the long-term effects of vaginal surgery and endogenous ovarian hormones on tissue function and ECM of the vagina and bladder in a rodent model. Most animal studies in the literature on vaginal surgery healing with or without sex hormones focus on short- to midterm recovery, between 7 and 42 days,[13,17-19] where changes are dominated by inflammatory responses. Hormone manipulation in these studies vary from ovariectomy to exogenous estrogen therapy or both. A recent meta-analysis of the effects of estrogen on vaginal wound healing ultimately included only 2 human and 12 animal studies that met selection criteria out of the 1474 articles that were screened.[11] The median duration of estrogen therapy in the selected studies was 14.3 days, and there was no reporting on the duration of follow-up. This underscores the lack of long-term data in this area of research.

Our study revealed significant long-term differences in tissue contractility, ECM gene and protein expression, and morphology among the experimental groups (with and without ovariectomy/vaginal surgery). The presence of circulating endogenous ovarian hormones mitigated many of the negative effects of surgery on tissue function and ECM, while the absence of hormones combined with surgery resulted in the most negative effects on function and ECM. In addition, expression of the peripheral nerve marker PGP9.5 declined in all surgical groups regardless of the presence or absence of ovarian hormones as compared with intact controls.

Notably, we found that in the vagina, ovariectomy was necessary to significantly reduce long-term contractility. Surgery alone was not significantly different from intact controls. Similarly, surgery alone did not significantly affect elastin protein expression when compared with intact controls. Interestingly, Pessina et al examined estrogen receptors in the rodent vagina and found that ovariectomy was associated with a significant increase in estrogen receptor alpha cells in all layers of the vaginal wall. Androgen receptors were found at very low levels in the vagina of controls and ovariectomized rats.[20]

Ovariectomy and vaginal surgery demonstrated significant downregulation in mRNA and protein expression of collagen I and III in the vagina as compared with intact controls, whereas vaginal surgery alone remained similar to controls. Our data are consistent with those of Callewaert et al, where they observed an increased ratio of collagen I to III in the vagina of a vaginal dilation injury rodent model at 42 days.[13] Reduction of certain ECM components in the ovariectomized rat after vaginal surgery can be attributed to a reduced estrogen level,[21] which may lead to impaired wound healing and altered growth factor signaling.[22,23] We saw no change in mRNA expression of smooth muscle markers (SMA and SMT), despite functional data showing that smooth muscle contractile responses are significantly lower with ovariectomy and vaginal surgery. This suggests that the decrease in function may not due to muscle atrophy but rather the remodeling of the ECM and its effect on smooth muscle cell connectivity.[24] Additionally, protein expression analysis revealed differences in elastin in the vagina among the groups, further exposing restructuring events in the ECM after surgery in the absence of ovarian hormones.

The effect of surgery and ovarian hormones was different in the bladder. KCl contractility of the dome and trigone was not significantly diminished by ovariectomy. However, there was a decrease in ECM protein expression in surgery with or without ovariectomy. The dome and trigone demonstrated a strong effect of surgery, with significant reductions in elastin and collagen III expression.

Vaginal surgery in the presence of intact endogenous ovarian hormones (V group) led to increased contractility in the trigone. Receptor-mapping studies have demonstrated that estrogen receptors are mostly identified in the trigone.[25-27] A low level of estrogen receptor alpha and beta staining has been documented in the bladder transitional epithelium and smooth muscle cells in controls and ovariectomized rats.[28] One hypothesis for the bladder trigone's distinctive contractile response stems from molecular studies finding that this region has higher expression of cellular adhesion, tight junction,[29] and gap junction markers[30] than the bladder dome. Gap junction protein—specifically, connexin 43 and 45 found in the bladder detrusor muscle and myofibroblast within the suburothelial layer—can enable rapid spread of electrical signals, which leads to synchronized contraction in smooth muscle fibers.[31-34] In response to injury or physical trauma, estrogen modulates the release of different cytokines, including tumor necrosis factor α, interleukin 6, and interleukin 10[35,36]; all of which have been linked to upregulation of connexin 43 in human bladder smooth muscle cells and suburothelial myofibroblast.[32] Additionally, inflammatory signals such as transforming growth factor ß are released in response to injury, inducing transition of fibroblast into myofibroblast-like cells.[37,38] In combination, this could result in more contractile cell types that synchronously produce the hypercontractile response seen in our data. This may also account for the increase in lower urinary symptoms reported by patients after surgery.[39] Future studies will investigate the potential role of this genetic and protein regulation and how it interacts with sexual function.

Although the difference was not consistently significant, ovariectomy and vaginal surgery did yield weaker dome contractions as compared with ovariectomy alone in response to high dosages of carbachol stimulation, suggesting the deleterious effect of surgery. Statistical significance between intact controls and other groups may be affected by the lack of control for hormonal fluctuation in the cycling intact rats. However, we believe that this effect is minimal since the SEM is similar in magnitude for the intact and ovariectomized groups. The bladder dome has a higher gene expression of muscarinic cholinergic receptor than the bladder trigone,[29] which may explain a different contraction pattern when stimulated by carbachol. Several studies on the effect of ovariectomy document a significant decrease in whole bladder contractions[40,41]; our study revealed that the combination of vaginal surgery and ovariectomy caused long-term functional and structural deficiencies in the bladder dome.

Taken together, our organ bath contractility data suggest that endogenous ovarian hormones may be able to rescue the negative effect of vaginal surgery, whereas the combination of ovarian hormone deprivation and vaginal surgery results in chronic deficiencies in function and ECM proteins.

Although neuromuscular function was not examined in this study, we were able to visualize the nerve network in tissues using immunohistochemistry stain for PGP9.5, a general neuronal marker, and quantify PGP9.5 mRNA expression in tissues. PGP9.5-positive staining was observed in the vaginal

and bladder epithelium and in the lamina propria. PGP9.5 mRNA expression and tissue stain locations declined in all surgical groups regardless of the presence or absence of ovarian hormones as compared with intact controls, suggesting that endogenous ovarian hormones may not be a significant factor in this observation. This is consistent with published rodent studies on the effect of estrogen on the vagina where ovariectomy or subsequent treatment with estradiol failed to produce any notable change on PGP 9.5 immunohistochemistry stains.[15] Future studies are needed to evaluate whether these observations correlate with changes in vaginal sensation.

A major limitation of this study is that it was done in a rat model. This limits our ability to extrapolate data to humans, where tissue healing is modulated by many factors. We also note that the RhoA/ROCK pathway, which is regulated by sex hormones, has been documented to be involved in vagina and bladder smooth muscle activity. However, due to an insufficient tissue sample, we were not able to evaluate this pathway in this study.

Conclusion

Our animal model showed that vaginal surgery and endogenous ovarian hormones can have significant long-term effects on the function and ECM of the vagina and bladder. Importantly, the presence of endogenous circulating ovarian hormones appeared to mitigate the long-term negative effects of vaginal surgery. The combination of endogenous ovarian hormone deprivation and vaginal surgery resulted in chronic negative effects on function and ECM. These findings contribute to our understanding of how hormonal factors moderate long-term tissue recovery.

Author contributions

T.H.: writing, conceptualization, methodology, visualization, data curation and analysis. J.Z., Y.W., S.G.: conceptualization, methodology, data curation and analysis. A.D.: conceptualization. B.C.: conceptualization, supervision, data analysis, writing, final approval of the manuscript.

Supplementary material

Supplementary material is available at *Sexual Medicine* online.

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Conflicts of interest

The authors declare no conflict of interest in relation to the content of the data presented.

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