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ORIGINAL STUDY

The ewe as an animal model of vaginal atrophy and vaginal Er:YAG laser application

AQ1 *Lucie Hympanova, PhD,[1,2,3] Rita Rynkevic, PhD,[1,2,4] Marina G. M. C. Mori Da Cunha, PhD,[1,2] Chantal M. Diedrich, MD,[5] Silvia Blacher, PhD,[6] Laurent De Landsheere, PhD,[7] Katerina Mackova, MD,[1,2,3] Ladislav Krofta, CSc,[3] Jan-Paul Roovers, PhD,[5] and Jan Deprest, PhD[1,2,8]*

Abstract

Objective: In sheep of reproductive age, we aimed to document decrease in epithelial thickness, glycogen amount, and other vaginal changes after castration and the effect of Er:YAG laser as used clinically.

Methods: On day 0, 16 sheep underwent ovariectomy. They were randomized to sham or three vaginal Er:YAG laser applications at monthly intervals. Primary outcome was vaginal epithelial thickness (d60, d71, d73, d77, and d160). Secondary outcomes included indicators of atrophy (vaginal health index = VHI), pH, cytology, morphology at the above time points, microcirculation focal depth (FD; d70 and d160), and at sacrifice (d160) vaginal dimensions and active and passive biomechanical testing.

Results: Menopausal changes between 60 and 160 days after ovariectomy included a progressive decrease in epithelial thickness, in VHI, FD, glycogen, elastin content and vasculature, and an increase in pH and collagen content. In lasered animals, the first day few white macroscopic foci were visible and an increased pH measured. Both disappeared within 3 days. Seven days after laser the epithelial thickness increased. At sacrifice (d160), there were no differences between sham and laser group in vaginal dimensions, morphometry, mitotic and apoptotic activity, active contractility, vaginal compliance, except for a lower blood vessel density in the lamina propria of the midvagina in laser group.

Conclusions: In reproductive sheep, ovariectomy induces vaginal atrophy evidenced in different outcome measurements. Vaginal Er:YAG laser induced visual impact, a short-term increase in epithelial thickness yet no long-term changes compared to sham therapy in menopausal controls.

Key Words: Biomechanics – Histology – Mini-invasive treatment – Ovariectomy – Sheep – Vaginal rejuvenation.

***Video Summary*:** http://links.lww.com/MENO/A672.

Vulvovaginal atrophy (VVA) is characterized by thinning of the vaginal epithelium and other layers, a decrease of glycogen and vaginal secretion, decrease in *Lactobacillus* spp. and transition to increase in pH.[1] These as other changes result in a more fragile vaginal wall, pain and increased risk of vaginitis.[1] VVA typically affects postmenopausal, but also young women with either early ovarian failure or following ovariectomy. VVA affects the quality of life in women with early menopause.[2] When VVA occurs in the context of endometrial- or breast cancer, systemic estrogen therapy is contra-indicated. Although vaginal estrogen therapy is widely considered safe, many women prefer, or their doctors advise them to avoid hormonal treatments.[3] Laser therapy is for these women an alternative,

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AQ2 From the [1]Centre for Surgical Technologies, KU Leuven, Leuven, Belgium; [2]Department of Development and Regeneration, KU Leuven, Leuven, Belgium; [3]Institute for the Care of Mother and Child, Third Faculty of Medicine, Charles University, Prague, Czech Republic; [4]INEGI, Faculdade de Engenharia da Universidade do Porto, Porto, Portugal; [5]Department of Obstetrics and Gynaecology, Amsterdam University Medical Center, Amsterdam, The Netherlands; [6]Laboratory of Tumor and Development Biology, GIGA-Cancer, Institute of Pathology, University of Liège, Liège, Belgium; [7]Department of Obstetrics and Gynaecology, University of Liège, Liège, Belgium; and [8]Pelvic Floor Unit, University Hospitals KU Leuven, Leuven, Belgium.

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Address correspondence to: Jan Deprest, PhD, Herestraat 49, 3000 Leuven, Belgium. E-mail: Jan.Deprest@uzleuven.be AQ3

TABLE 1. *Timeline of procedures and assessments.*

Days after ovariectomy	0	60	70	71	73	77	100	130	160
Surgical interventions									
Bilateral ovariectomy	X								
Sham or laser			X				X	X	
Biopsy/necropsy		X		X	X	X			X
Minimally invasive outcome measurements									
pH		X		X	X	X			X
Vaginal health index		X		X	X	X			X
Microcirculation focal depth		X							X
Vaginal cytology		X		X	X	X			X
Histology		X		X	X	X			X
Terminal measurements									
Active biomechanics									X
Passive biomechanics									X

inspired by earlier successful application for skin resurfacing.[4] The most common laser sources are CO_2 and Er: YAG, yet they can be used with a variety of energy, mode, and settings. The targeted tissues include the vaginal epithelium and the lamina propria.[5] An energy beam can be either a full field or delivered fractionally, that is an array of multiple smaller spots of application. In the nonablative mode light energy is locally transformed to heat, inducing a tissue reaction characterized by an increase in fibrillar diameter of the collagen already present[6] and stimulation of neo-collagenesis[7] without superficial injury. The surrounding tissue can participate in faster healing and remodeling.[5] In a controlled cohort study nonablative fractional Er: YAG laser application improved VVA[8] and in noncontrolled studies vaginal relaxation syndrome and mild forms of incontinence or prolapse.[8,9] Vulvo-vaginal laser is however novel and there is a lack of experimental studies that document the exact histologic and biomechanical response to Er:YAG laser.

For better insight into the working mechanism and optimized setting of laser therapy, we aimed to develop an animal model in sheep. The ewe is a large animal model to study the systemic effects of menopause such as hot flushes,[11] osteoporosis,[12] or cardiovascular disorders.[13] We have used the model for simulating physiologic changes in the pelvic floor, such as vaginal birth, iatrogenic menopause, and systemic hormonal therapy.[14] We therefore thought this was a good model to document the longitudinal changes induced by ovariectomy in reproductive multiparous sheep. We measured these at the clinical, histological, and biomechanical level.

METHODS

Animals and study design

Sixteen Swifter multiparous ewes were scheduled to undergo ovariectomy (d0) (anesthesia and protocol are in the Supplementary Material, http://links.lww.com/MENO/A673). All further procedures were performed under sedation by xylazine 10 mg/kg IM (Xyl-M; VMD; Arendonk; Belgium).[15] At d60 they underwent a baseline qualitative vaginal assessment, consisting of vaginal inspection, cytology, vaginal microcirculation focal depth (FD) measurement and biopsies. Then they were randomized to either undergo a sham or vaginal laser procedure on d70 (Table 1). Vaginal assessments were repeated 1 (d71), 3 (d73), and 7 (d77) days after laser. Additional laser or sham applications were done 1 and 2 months later (d100 resp. d130), as clinically suggested. Finally, animals were euthanized d160.

Anatomical assessment

Vaginal atrophy was scored by one observer blinded to the evaluated group with the Vaginal Health Index (VHI).[16] The VHI is a clinical noninvasive semiquantitative assessment of vaginal elasticity, fluid volume, moisture, epithelial injury, and pH (Table 2). The vagina was also inspected for abnormalities as erythema, petechiae, bleeding, inflammation, erosion, edema, and scarring. A vaginal wet smear with a saline soaked Q-tip was taken before taking biopsies. The swab was rolled over a slide glass, allowed drying, stained with Papanicolaou, and quantified for the presence of parabasal, intermediate, and superficial cells.[17] Clinically, less than 5%

TABLE 2. *Vaginal Health Index[15] as used in this experiment*

Score	1	2	3	4	5
Elasticity	None	Poor	Fair	Good	Excellent
Fluid Volume	None	Scant amount, vault not entirely covered	Scant amount, vault entirely covered	Moderate amount	Normal amount
pH	9 and more	8.5	8	7.5	7 and less
Epithelial injury	Petechiae noted before contact	Bleeds with light contact	Bleeds with scraping	Not friable-thin epithelium	Normal
Moisture	None, surface inflamed	None, surface not inflamed	Minimal	Moderate	Normal

The clinical pH scale was adjusted to the findings in menopausal sheep. Reproductive ewes have a vaginal pH of 6.7 ± 0.4.[17] Postmenopausal sheep had a higher pH, ranging from 6.6 up to 9.0.

superficial cells indicates vaginal atrophy.[17] The pH was measured by two methods. The first semiquantitative method using pH strips (Sigma-Aldrich, Diegem, Belgium; range 7-14, resolution 0.5) was necessary as it is part of the VHI. A second quantitative biochemical test was done with a pH-meter-1000L (VWR, Leuven, Belgium; range: −2 to +20; resolution 0.001).[18]

Biopsies

The vagina was locally disinfected with natriumhypochlorite 0.5 g/100 mL (Melisana, Brussels, Belgium) and anesthetized with lidocainhydrochloride gel (Xylocaine, AstraZeneca, Brussels, Belgium). A biopsy (ø5 mm) was taken using an Acu-Punch (Acuderm, Fort Lauderdale, Florida) biopsy device. To avoid overlap, biopsies were taken consecutively in the four quadrants along a circular line 1 cm above the hymen (Fig. 1A, B) and fixed in paraformaldehyde 4%.

Microcirculation focal depth measurement

The Incident Darkfield Illumination technique visualizes erythrocytes by green light absorbed by hemoglobin.[19] A handheld microscope Cytocam-IDF (Braedius Scientific BV, Huizen, The Netherlands) was used to measure the distance between the epithelial surface and the most proximal microcirculation layer of the lamina propria (4 μm accuracy).[20] The measuring probe was placed 3 cm cranial from the hymen, perpendicular to the vaginal wall with measurements taken in the four quadrants (Fig. 1C). Three measurements per location were performed and an average focal depth was calculated. Measurements were taken at baseline and at the end of the experiment.

Sham or laser procedure

The circular adaptor and a full-field collimated handpiece (ID88920 resp. R11, Fotona, Ljubljana, Slovenia) were inserted (Fig. 1D, E). Afterward either laser energy was applied, or in sham, no energy. The laser application followed the nonablative SMOOTH IntimaLase protocol as clinically used.[8] This provided deposition of energy to the lamina propria (200 μm under the surface)[21] to the full circumference of the entire vaginal canal[9] (wavelength 2,940 nm, spot size 7 mm, fluence 3 J/cm^2, 4 pulses, ejection steps distance 5 mm, five passes along the entire canal).[8]

Obduction

The ewes were euthanized by intravenous administration of pentobarbital (Release, Ecuphar, Oostkamp, Belgium 20 mL/50 kg). After vaginal assessment, the vagina was dissected, measured, and evaluated macroscopically after a standardized protocol.[22] Three specimens were taken from the distal and middle vagina per animal (Supplementary

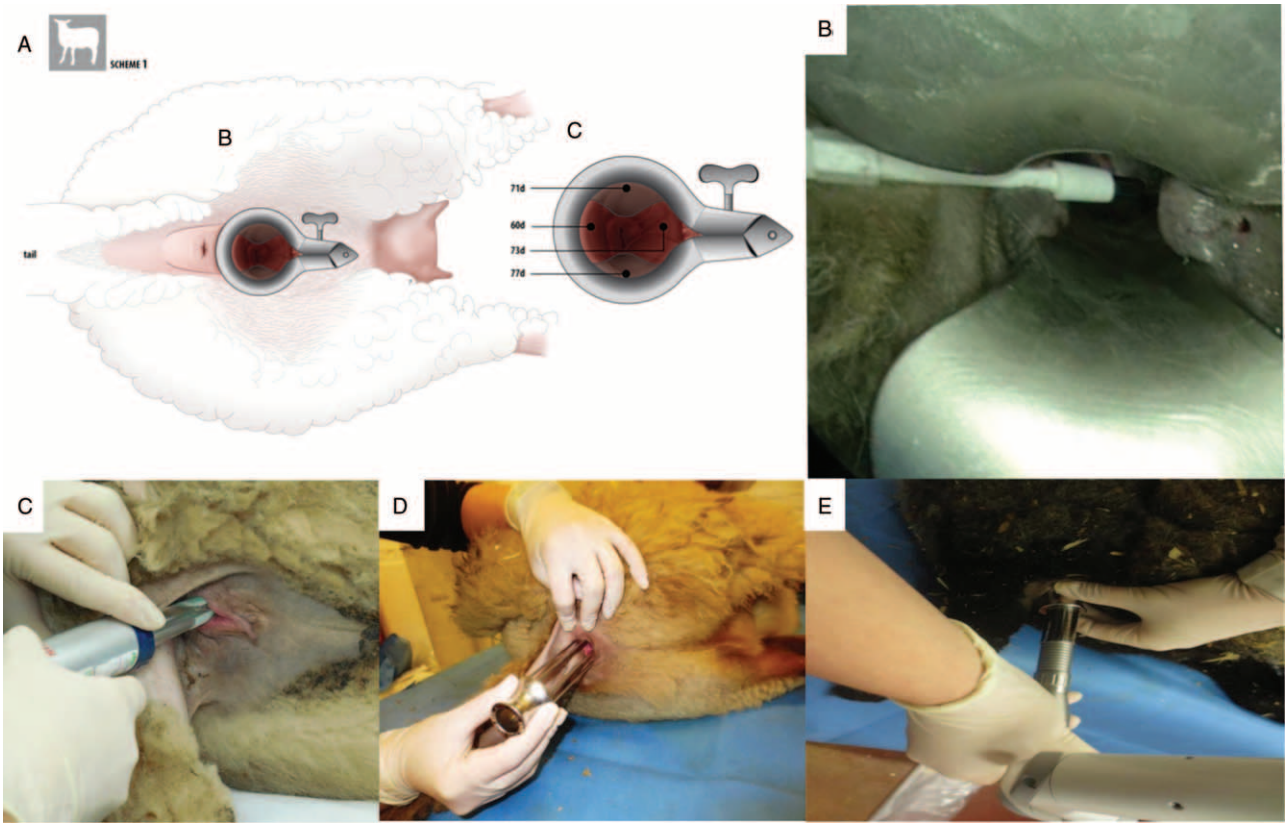

FIG. 1. (**A**) Schematic figure of locations, where biopsies were taken (**B**) real vaginal punch biopsy day 73 (**C**) Cytocam microcirculation focal depth measurement (**D**) vaginal insertion of a circular adaptor ID88920 and (**E**) laser delivering handpiece R11 inserted in adaptor.

Figure 1, http://links.lww.com/MENO/A674) for active and passive biomechanical testing and histology.

Active and passive biomechanics

Vaginal contractility was measured as previously reported[23] (Supplementary Material, http://links.lww.com/MENO/A673). Briefly, freshly harvested vaginal strips (4×8 mm) were placed into baths filled with Krebs solution, preloaded and let to equilibrate for 1 hour. Then they were exposed to a gradually increasing amount of potassium chloride (10-120 mM). Contractile forces and time were recorded by custom-made software and analyzed using Origin-software (Origin Lab Corporation; Northampton, MA). Values were normalized to sample weight, gravitation constant (9.81), a transducer calibration-factor and the baseline contraction was subtracted. Contraction at 80 mM KCl was compared between groups.

For passive biomechanical testing, 35×35 mm specimens were stored in saline soaked gauzes at −20°C till testing. They were thawed 6 hours at room temperature before testing and clamped in a Zwick tensiometer (Zwick GmbH & Co. KG, Ulm, Germany) ball-burst setup (11.5 mm plunger, ø30 mm compression ring, 20 mm aperture, preload 0.1N, speed 10 mm/min).[24,25] Force–displacement curves were recorded till disruption or reaching the maximal cell load of 200N by TestExpert-software (Zwick). Stiffness was calculated using custom-made Excel (Microsoft Office, Redmond, WA) and normalized to the specimen thickness.

Histology, immunohistochemistry, and immunofluorescence

Two observers blinded to the treatment groups evaluated the biopsies and explants. From each specimen up to 10 photographs were taken randomly, the actual number depending on specimen size. Details on staining are in the Supplementary Material, http://links.lww.com/MENO/A673. Hematoxylin and Eosin (H&E) slides were examined for surface injury, epithelial thickness (μm), minimum and maximum count of epithelial cell layers and the count of papillae.[26] Masson's Trichrome and Miller were used to semiquantify collagen and elastin in the lamina propria.[27] Periodic acid-Schiff (PAS) visualized epithelial glycogen accumulation.[28] Vascularity was quantified by endothelial cell marker CD34, nerves by protein gene product 9.5 (PGP9.5). Ki-67 and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) quantified proliferation and apoptosis in the epithelium and lamina propria. Fluorescent images were acquired using the Leica DM5500 microscope (Leica Microsystems, Wetzlar, Germany). A comprehensive evaluation of the full specimen at smaller magnification was done for explants including density of collagen, elastin and smooth muscle (alpha smooth muscle actin; α-SMA),[29] and the thicknesses of the epithelium, lamina propria, and muscularis (μm).

Statistics and ethics

Experiments were approved by the Ethics Commission on Animal Experimentation of the KU Leuven. Normality testing was done by the D'Agostino Pearson test. Comparison of quantitative data was performed with an unpaired *t* test for normally distributed data and Mann-Whitney for non-normally distributed data. Longitudinally collected data were tested by mixed effects model. According to normality data were reported as mean ± SD or median and interquartile range. The statistical significance level was defined as $P < 0.05$. This experiment was powered based on findings in a previous study [14] and an expected difference in epithelial thickness of 58.7% (Details Supplementary Material, http://links.lww.com/MENO/A673).

RESULTS

Longitudinal observations in menopausal sheep

All outcome values are displayed in Table 3. From 60 to 160 days after ovariectomy the VHI, FD decreased and pH increased (Fig. 2). Under the microscope we observed epithelial thinning and glycogen loss (Fig. 3). Changes of vaginal cytology were not significant, however supporting vaginal atrophy by decreased percentage of superficial cells. We also observed increase in collagen, decrease in elastin and vascularization. We did not detect changes in innervation, mitotic, and apoptotic activity.

Laser application

Technically the laser application was feasible without complications. There were a few irregularly dispersed white foci (<5 mm) on the vaginal surface visible d1 postlaser, which disappeared by d3. The pH was higher d1 after laser therapy as compared to sham as well as to baseline (Fig. 4). Later pH measurements fell within the range of the sham animals. The VHI and cytology were also comparable to observations in shams. Day 7 the epithelium was significantly thicker in the laser group (laser 93.0 ± 3.6 μm vs sham 71.4 ± 5.2 μm; $P = 0.05$). Microscopically, there were no local signs of heat injury in the laser group at the predefined locations where the biopsies were taken. We did not have biopsies from the white foci as these were out of predefined biopsy areas. Vascularization, innervation, proliferation, and apoptosis were comparable in both groups at each time point, except at 160 day in the middle vagina lasered explants had a lower vascular count.

Additional morphologic and biomechanical findings in the specimens taken only at obduction showed no differences in vaginal dimensions (laser 80.5 ± 3.9 × 34.8 ± 1.8 vs sham: 81.0 ± 3.6 × 34.0 ± 1.1), contraction and stiffness (Supplementary Figure 2, http://links.lww.com/MENO/A675). Morphometry on full-slide, thickness of the vaginal epithelium, lamina propria, and muscularis were comparable, just as the density of collagen, elastin, and smooth muscle (Supplementary Figure 2, http://links.lww.com/MENO/A675, Table 3).

DISCUSSION

This study showed that castration in the ewe results in vaginal changes that reflect the effects of menopause in humans. The effects of vaginal laser therapy on the vagina

TABLE 3. *All outcome variables from punch-biopsies (days 60, 71, 73, 77; in the distal vagina) and at terminal point (160 days; both in the distal and middle vagina)*

	Distal vagina										Middle vagina	
Day after ovariectomy	60 d		71 d		73 d		77 d		160 d		160 d	
Group	Sham	Laser	Sham	Laser	Sham	Laser	Sham	Laser	Sham	Laser	Sham	Laser
Epithelial thickness (μm)	76.0 ± 3.8 b	65.7 ± 16.5 b	87.6 ± 6.2	80.7 ± 11.2	109.5 ± 7.5	101.4 ± 7.2	71.4 ± 5.2 a	93.0 ± 3.6 a	30.5 (2.29-71.7) b	38.7 ± 6.6 b	33.5 ± 2.6	36.6 ± 6.3
Epithelial layers (max)	7.0 ± 0.5	6.0 ± 0.7	6.5 ± 0.2	6.0 ± 0.4	7.0 ± 0.5	6.5 ± 0.5	6.0 (5.0-6.8)	6.5 ± 0.3	5.0 ± 0.7	5.0 ± 0.4	5.0 ± 0.5	5.0 (5.0-6.0)
Epithelial layers (min)	3.0 ± 0.3	3.5 ± 0.5 b	3.0 (2.5-3.0)	2.0 ± 0.3	3.0 (3.0-4.0)	2.5 ± 0.3	2.5 ± 0.3	2.0 ± 0.3	2.0 ± 0.3	2.3 ± 0.2 b	2.0 ± 0.2	2.0 ± 0.3
Papillae (count)	0 (0.0-0.0)	0 ± 0.9	0 ± 0.2	0 (0.0-0.0)	0 (0.0-1.0)	0 (0.0-0.0)	0 ± 0.3	0 (0.0-0.0)	0 ± 0.5	0 (0.0-0.0)	0 (0.0-0.0)	0 (0.0-0.0)
Collagen (%area)	50.2 ± 4.9	38.0 ± 2.4b	41.1 ± 6.1	42.0 ± 6.1	41.9 ± 4.2	43.6 ± 4.0	30.5 ± 4.0	35.1 ± 5.6	59.9 ± 6.6	59.3 ± 4.4b	68.1 ± 3.3	70.3 ± 4.6
Elastin (% area)	6.6 ± 5.1b	8.4 (3.2-9.2) b	3.5 ± 1.7	5.4 ± 2.8	3.8 ± 2.0	3.3 ± 1.7	2.9 ± 1.0	2.6 ± 1.7	2.2 ± 0.9 b	2.3 ± 1.3b	2.0 (1.1-3.0)	2.3 ± 1.8
PAS (μm)	10.3 (9.1-29.5) b	8.9 ± 1.5 b	12.4 ± 0.8	13.0 ± 1.8	8.8 ± 0.8	7.1 ± 1.1	6.5 ± 1.4	6.7 ± 0.4	2.4 ± 0.4 b	3.2 ± 0.7 b	2.2 ± 0.4	2.5 ± 0.3
CD34 (%area)	11.9 ± 2.2 b	10.5 ± 1.3 b	9.9 ± 1.2	9.8 ± 0.8	6.0 ± 0.7	3.4 ± 1.0	8.8 ± 0.5	6.1 ± 1.0	5.6 ± 1.8 b	5.1 ± 0.9 b	3.0 ± 0.3 a	2.3 ± 0.3 a
PGP 9.5 (%area)	0.4 ± 0.2	1.0 ± 0.7	0.7 (0.4-0.8)	1.7 ± 1.1	0.2 ± 0.1	0.6 ± 0.3	0.4 ± 0.2	0.6 ± 0.2	0.5 ± 0.3	0.4 (0.3-0.8)	0.7 ± 0.3	1.2 ± 0.9
Ki67												
Epithelium	3.0 (2.4-5.0)	8.8 ± 10.7	31.0.5 ± 15.9	33.4 ± 12.7	10.0 ± 6.7	12.8 ± 6.2	3.2 (1.8-4.1)	2.2 (1.4-3.6)	2.3 ± 1.7	1.1 (0.3-1.8)	1.5 (1.0-2.6)	2.9 ± 2.3
Lamina propria	0.0 (0.0-0.5)	0.0 (0.0-0.4)	1.0 (0.4-1.4)	1.3 ± 1.1	0.6 ± 0.6	2.1 ± 1.7	0.4 (0.1-1.6)	0.3 (0.0-1.8)	0.2 ± 0.1	0.1 ± 0.1	0.2 (0.0.-0.2)	0.1 (0.0.-0.4)
TUNEL												
Epithelium	2.2 ± 1.5	0.6 ± 0.7	2.2 ± 1.0	3.0 ± 1.9	2.3 ± 1.8	1.4 ± 1.3	2.8 ± 2.2	0.3 (0.0.-0.9)	2.7 (1.6-4.7)	3.9 (1.9-5.9)	2.3 ± 2.7	6.9 ± 6.7
Lamina propria	0.6 (0.5-1.4)	0.3 ± 0.3	1.3 ± 1.2	1.4 (0.3-5.5)	0.6 (0.4-1.7)	0.9 ± 0.4	0.8 ± 0.7	1.2 ± 1.2	0.5 (0.2-1.4)	1.5 ± 1.3	0.6 ± 0.6	0.5 ± 0.7
VHI	17.8 ± 1.0b	18.4 ± 0.5b	18.0 (16.2-18.0) a	17.5 (17.0-18.8) a	17.2 ± 1.9	17.0 ± 1.5	18.1 ± 1.6	18.6 ± 1.1	15.5 ± 0.9b	16.4 ± 0.7b	-	-
pH meter	6.8 ± 0.1 b	7.0 ± 0.2	7.2 ± 0.1 a	7.6 ± 0.1 a	7.1 ± 0.1	7.0 (6.9-7.3)	7.0 ± 0.1	6.9 ± 0.1	7.7 ± 0.3 b	7.3 ± 0.2	-	-
Smear SPF (%)	7.7 ± 10.9	4.7 ± 5.6	4.0 ± 3.7	0.0 (0.0-2.0)	9.6 ± 7.5	9.3 ± 5.3	10.1 ± 8.4	5.5 (3.1-8.8)	0.0 (0.0-0.0)	0.0 (0.0-0.7)	-	-
Smear INT (%)	45.8 ± 18.9	48.0 ± 15.9	29.1 ± 12.3	41.6 ± 14.5	37.2 ± 11.5	42.3 ± 15.3	44.8 ± 17.6	35.8 ± 18.9	43.8 ± 20.8	36.8 ± 23.4	-	-
Smear PB (%)	46.5 ± 23.9	39.6 ± 17.8	67.0 ± 12.7	56.7 ± 13.9	53.3 ± 11.8	57.0 (39.4-65.8)	45.1 ± 19.7	49.2 ± 24.4	54.0 ± 19.9	53.8 ± 26.0	-	-
Vaginal focal depth	140.0 (132.0-156.0) b	142.5 ± 19.0 b	-	-	-	-	-	-	60.0 (47.8-80.0) b	67.0 (5.0-80.0) b	-	-
Comfort zone stiffness (N/mm)	-	-	-	-	-	-	-	-	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.3	0.6 ± 0.2
Contractility	-	-	-	-	-	-	-	-	119.4 ± 48.5	100.9 ± 44.9	167.8 (96.9-188.7)	189.5 ± 81.4
Density (%area)												
Collagen	-	-	-	-	-	-	-	-	0.61 (0.60-0.67)	0.63 ± 0.11	0.62 ± 0.07	0.70 ± 0.08
Elastin	-	-	-	-	-	-	-	-	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
Smooth muscle	-	-	-	-	-	-	-	-	0.18 ± 0.08	0.22 ± 0.06	0.20 ± 0.07	0.18 (0.15-0.18)
Thickness (mm)												
Epithelium	-	-	-	-	-	-	-	-	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Lamina propria	-	-	-	-	-	-	-	-	0.56 ± 0.18	0.52 ± 0.16	0.42 ± 0.11	0.55 ± 0.28
Muscularis	-	-	-	-	-	-	-	-	2.17 ± 0.68	1.98 ± 0.63	2.04 ± 0.48	2.21 (2.02-2.39)

The letter (a) marks a significant difference ($P < 0.05$) between sham and laser group at given time-points, letter (b) marks a significant difference when 60 and 160 d (longitudinal comparison) are compared within the sham or laser group. Collagen (Masson's Trichrome), elastin (Miller), blood vessels (CD34), and nerves (PGP9.5) were measured as area stained. Cells undergoing mitosis (Ki-67) and apoptosis (TUNEL), epithelium cell layers, papillae and superficial (SPF), intermediate (INT), parabasal (PB) cells were manually counted. Apoptosis and mitosis were assessed in two layers, the epithelium (Epit.) and lamina propria. The pH reported in the table was measured by pHmeter. Full slide measurements at low magnification were performed only from terminal explants and they are reported at the end of table together with stiffness and contraction. Normally distributed data are reported as mean ± SD, not normally distributed as median (interquartile range: P25-P75).
CD34, cluster of differentiation 34; Ki67, protein MKI67; PAS, periodic acid Schiff; PGP 9.5, Protein gene product 9.5; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VHI, vaginal health index.

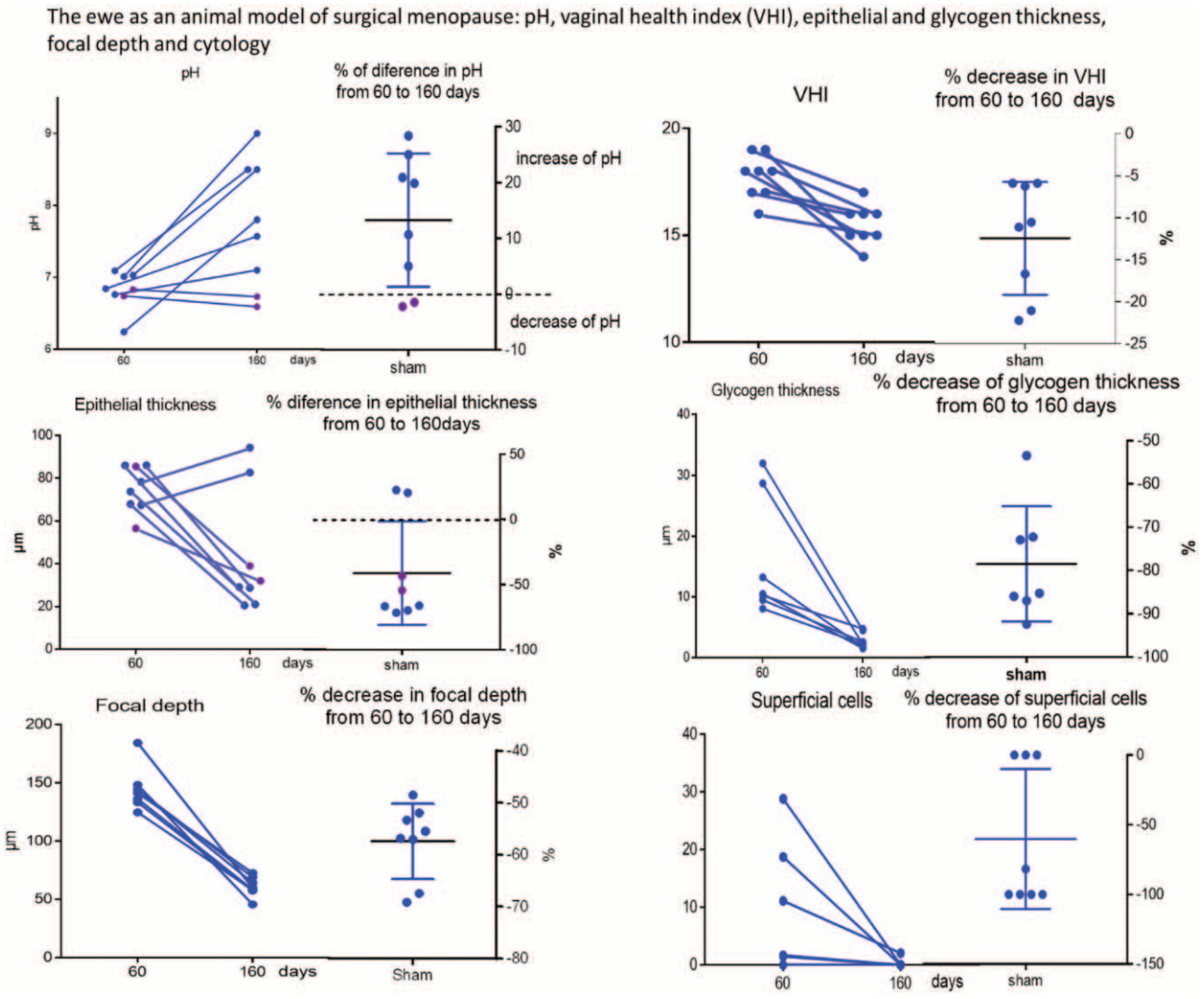

FIG. 2. Ewe as animal model of surgical menopause (sham group). Displaied are changes from 60 to 160 days: pH, vaginal health index (VHI), epithelial and glycogen thicknesses, vaginal microcirculation focal depth (FD), and cytology (superficial cells). Each readout is on a left side of graph displied as individual changes from 60 to 160 days conected with line and on the right side as a % diference between 60 and 160 days, each dot represents one animal. Most of the animals had increase of pH (blue dots) only two animals did not have change (purple dots), those were not same animals as those, which did not have change of epithelial thickness (purple dost in graph of epithelial thickness).

of the ewe were limited and reversion of castration-related changes was not observed.

Vulvovaginal atrophy is a syndrome with a wide variety of changes in the urogenital tract. One of the hard outcome measures is thinning of the vaginal epithelium. In premenopausal women ranges of 211 to 280 µm are reported,[30] falling to 170 to 200 µm at 1 year after the last menstruation.[31] In premenopausal ewes an epithelial thickness ranging from 40 to180 µm was previously reported.[32,33] In a previous experiment we observed 35.8 ± 24.7 µm in nulliparous reproductive sheep, which is marginally different. This fell to 14.0 ± 10.4 µm 60 days after ovariectomy.[14] In this experiment we measured different absolute values, however also a significant drop, that is from 76.0 ± 3.8 µm 60 days after castration to 30.5 ± 10.1 µm three months later. These differences in absolute values between experiments may be because ewes may not have been fully or long enough menopausal at the start of the experiment. This is additionally supported by the observations on vaginal cytology. Some animals (7/16) had 60 days after ovariectomy still more than 5% superficial cells, but 160 days after ovariectomy all ewes had less than 5%.

We documented several other parameters that correlate with clinical measurements, like VHI, which decreased between 60 and 160 days after ovariectomy. In retrospect, as a novel parameter first evaluated in sheep, we should have included VHI measurements at the start of the experiment, when the animals were premenopausal. We have done so in other experiments with nonsynchronized premenopausal sheep of 1 to 2 years old, where the VHI was at least one-third higher (24.0 ± 1.0; $P < 0.001$; data not shown). The analogy to the human vaginal health index is only partly

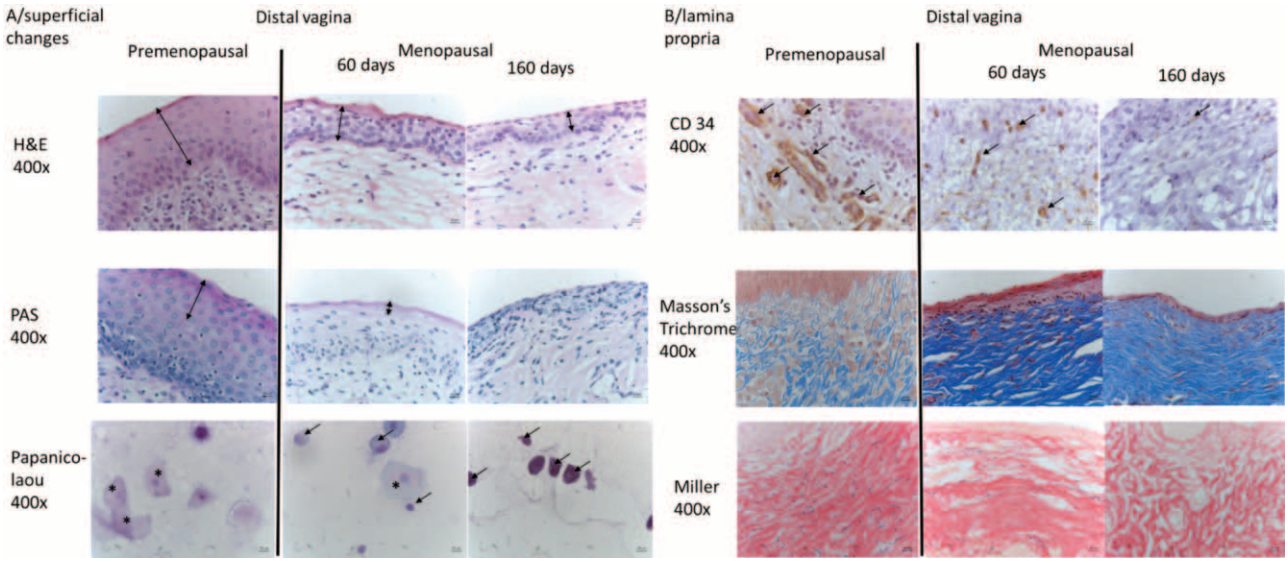

FIG. 3. Representative figures of changes in A/ epithelium and B/ lamina propria. From left to right, first are figures from premenopausal sheep (different experiment), futher 60 and 160 days postovariectomy, all figures are distal vagina of sham group, magnification ×400. On H&E arrows mark thicknesses of epithelium and on PAS glycogenized epithelium, at 160 days in some areas the glycogen was completely lacking as shown on figure. At 160 days, vaginal cytology (papanicolau stain) is visible predominance of parabasal cells (arrows) and lacking of superfial cells(asterisk) which are frequent premenopausaly. At CD34 (stain brown) arrows mark vessels. Connective tissue, mainly collagen (stained blue) at Masson's Trichrome and elastin (black lines) at Miller stain.

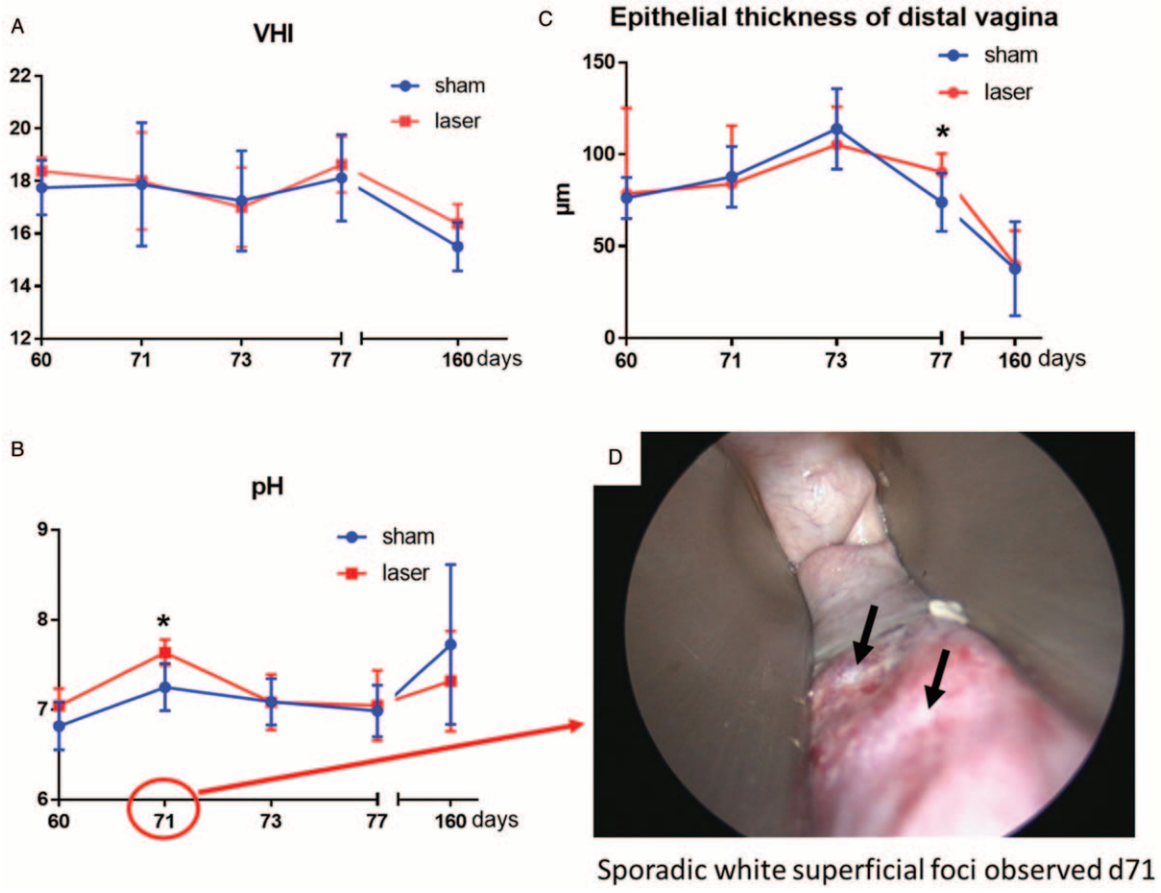

FIG. 4. (**A**) Vaginal health index, (**B**) pH, and (**C**) epithelial thickness, all measured five times; prior first laser aplication (d60), first, second, and third day after and at the end of experiment 160 days. pH was significantly different between laser and sham group day 71, when also sporadic white foci (D) were observed (arrows). (**C**) Epithelial thickness, where diference between sham and laser group was observed at day 77. At graphs, measurements are displaied as mean ± SD, asterisk marks significance ($P < 0.05$).

correct, as the *absolute* pH of the ovine vagina is remarkably higher at any stage (Premenopausal: 6.70 ± 0.38).[18] This may be due to different reasons, for example, by a different vaginal flora. In women, *Lactobacillus* spp. are abundantly present causing women having the lowest vaginal pH during reproductive life (3.8-4.5) of all mammals. *Lactobacillus* spp. are present in 80% of ewes, but their number is very low, potentially explaining the higher pH.[18,34] The pH of the sheep in our experiment was high at baseline (mean pH = 6.8) and increased to an average of pH = 7.7.

FD was already once used to noninvasively document vaginal thickness in women with VVA. FD of those women with atrophy was 80 μm [interquartile range 80-120 μm] with a significant increase after local estrogen application to 220 μm [148-248 μm].[20] We present the first study using FD in sheep. Again unfortunately no baseline precastration values were measured, however also this parameter showed a progressive decline in FD between 60 and 160 days. We neither have any premenopausal reference values, as at that time we did not have full time access to the hardware.

We were also interested in changes in the lamina propria. In our previous study, 60 days after ovariectomy we observed an increase of collagen and elastin,[14,35] as did others.[36] Increased collagen content is in line with clinical observations.[37] In the present study, between 60 to 160 days after ovariectomy, we also observed an increase in collagen that was only significant in the laser group. As to elastin the situation is different. Also at 60 days, the variability in elastin was rather high, yet by 160 days that range is narrower and its values being low. The present histological findings are in line with clinical loss of vaginal elasticity in menopausal women.[37] This experiment was also designed to study changes induced by Er:YAG laser with a protocol clinically used for VVA. To our knowledge, four studies have described histological changes in the vaginal wall after Er:YAG laser application. All are on rather small sample sizes. Three studies focused on the treatment of stress urinary incontinence,[38-40] one on VVA.[41] The last reports an increase in epithelial thickness, an increased number of papillae, cellularity and neo-angiogenesis in the lamina propria; however it was not quantified.[41]

In our experiment we found only a transient increase of epithelial thickness 1 week after initial laser application, and a decreased vascularization in the middle vagina at 160 days. Several explanations may be possible for this initial change to be not sustained. First, the energy settings and delivery protocol may not have been appropriate to induce more and durable changes. The epithelial thickness of the ewe is thinner compared to humans, therefore the energy could in ewes be transmitted deeper hence have been less effective superficially. We can only test this in a new experiment, using different settings and/or in premenopausal sheep. Another variable may be that the water content of the ovine vagina may be different from that in women, so that the clinical protocol we used was inappropriate for sheep. Water content is important as eventually the Er:YAG laser energy with its wavelength of 2,940 nm is mainly absorbed by water. We have no knowledge about the exact water content of vagina in ewes and how it compares to that of women. Another reason may be that the vagina was not (yet) atrophic enough. This could be because of external factors, such as the nature of their food intake prior to entering the experiment. Some plants, like red clover, have higher phytoestrogen concentrations.[42] This could in turn explain why at 60d following ovariectomy the ewes from this experiment had still a thicker vaginal epithelium than the ones in our previous experiment.[14] Along the same lines, the interval between ovariectomy and laser application might have been too short, as evidenced by the ongoing progress of changes between 60 and 160 days. Because our experimental results contrast with what is clinically claimed, we continue research to optimize settings of this vaginal atrophy animal model.3

We have to acknowledge several limitations to this experiment. First, the ewes used in this experiment were not aged hence not reaching natural menopause, and as a consequence we did not study *naturally* occurring VVA. Actually, sheep as many other animals never reach natural menopause. We herein induced menopause at the age of seven (natural life expectancy: 10-12 y) by castration. Ovariectomy in sheep unequivocally induces menopause with all systemic consequences observed in women (ie, cardiovascular disorders,[13] hot flushes,[43] or osteoporosis[12]), as this does also in women undergoing bilateral ovariectomy.[44] Another measure we could have taken in this respect is estrogen levels. We did not do so as 17 beta-estradiol levels below 1 pg/mL have been documented in sheep within 1 month following castration, persisting for 6 months at least.[45] Another limitation of this experiment is that most parameters are missing at baseline. Also, the interim time point of 60 days was not completely documented with all outcome measures. This was however a longitudinal experiment; only nonterminal outcome measures could be repetitively documented. Also, histology on vaginal biopsies is not similar to full-thickness specimens because they are not deep enough for evaluation of the lamina propria and muscularis.

This study also has strengths we would like to address. It is the first study documenting medium-term changes in the sheep vagina after iatrogenic menopause, documenting in detail vaginal atrophy and deeper tissue changes as well as its progression over time. It is also the first study on vaginal laser application in sheep, even though at these settings we did document many changes.

Potential Clinical Value: We will continue to optimize the ewe as a preclinical model for vaginal atrophy, as we believe that interventions like laser therapy should be evaluated in animals before exposing humans to it.

CONCLUSIONS

We showed that sheep after ovariectomy develop vaginal atrophy. We documented progression of the clinical expression of vaginal atrophy between 60 and 160 days after ovariectomy. Using the same outcome measurements we were not able to detect difference between Er: YAG and sham exposed ewes.

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