Levator ani muscle and connective tissue changes associated with pelvic organ prolapse, parity, and aging in the squirrel monkey: a histologic study

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OBJECTIVE: This study was undertaken to evaluate histologically the levator ani muscle and paravaginal attachments in squirrel monkeys with and without pelvic organ prolapse.

STUDY DESIGN: Serial sections from 19 females were processed with routine histology stains. Fiber typing was performed with antifast (type II) and antislow (type I) skeletal myosin antibodies, and apoptotic nuclei were examined by dUTP nick-end labeling (TUNEL).

RESULTS: Gross disruption of the levator ani muscle and its innervation was not observed in animals with or without visible support defects. Myogenic changes occurred more frequently in the pubocaudalis than iliocaudalis muscles, and a significant association was found with aging ($P < .05$, Fisher exact test) but not with pelvic organ prolapse or parity. Neurogenic changes were observed in 3 of 13 multiparous monkeys. Myocyte diameter increased in animals with pelvic organ prolapse compared with age-, weight-, and parity-matched animals without pelvic organ prolapse ($P = .005$) and correlated with levator ani muscle wet weight ($R = 0.76$; $P = .0006$). In the paravaginal attachments, the numbers of fibroblasts and apoptotic nuclei were not different between animals with and without pelvic organ prolapse, but parity was associated with increased apoptosis ($P = .025$).

CONCLUSION: Vaginal prolapse in the squirrel monkey does not result from atrophy or gross disruption of the levator ani muscle or its innervation. As in women, myogenic changes are a common finding in the levator ani muscle and increase with aging, whereas denervation with subsequent reinnervation occurs in some multiparous monkeys.

Key words: histology, levator ani, myogenic, neurogenic, pelvic organ prolapse, squirrel monkey

Cite this article as: Pierce LM, Baumann S, Rankin MR, et al. Levator ani muscle and connective tissue changes associated with pelvic organ prolapse, parity, and aging in the squirrel monkey: a histologic study. Am J Obstet Gynecol 2007;197:60.e1-60.e9.

Approximately 1 in 9 women undergo surgery for the correction of pelvic organ prolapse (POP) and associated bladder and bowel dysfunction.[1] Despite its common occurrence, the cause of POP remains largely unknown. Prolapse is thought to be caused by direct injury to the levator ani (LA) muscle, denervation of the pelvic floor musculature, or fascial damage incurred during childbirth trauma.[2] Defects in the fibromuscular connective tissues of the vaginal wall and supportive structures, and abnormal synthesis or degradation of collagen are also thought to be contributing factors.[3]

Neurogenic and/or myogenic damage have been reported in the LA muscle and external anal sphincter from patients with stress urinary incontinence, anal incontinence, and/or POP.[4-8] These striated pelvic floor muscles, like most skeletal muscle, express various metabolic phenotypes dependent on their location and contractile function and contain a mixture of type I (slow twitch) and type II (fast twitch) fibers.[4-10] Neurogenic changes, which are caused by denervation, are characterized by atrophy of groups of fibers that have lost their nerve supply, angular outline of atrophied fibers indented by surrounding fibers of either normal size or fibers that undergo compensatory hypertrophy, and fiber type grouping.[11] Fiber type grouping, an alteration in the normal mosaic pattern of skeletal muscle in which a large cluster of type I fibers is adjacent to a large cluster of type II fibers, occurs with partial denervation and subsequent reinnervation (ie, sprouting of remaining motor axons), because muscle fiber type is dependent on the type of motor neuron innervating the fiber.[11,12] Myogenic changes include variation in fiber size (with hypertrophic and rounded atrophic fibers), selective type II fiber atrophy (nonspecific myogenic change), increased numbers of fibers with central nuclei, and increased endomysial collagen and adipose tissue (indicative of chronic myopathy).[11] Chronic denervation may cause secondary myogenic changes, such as fiber necrosis, fiber hypertrophy and splitting, increased central nucleation, and eventually extensive fibrosis and fatty replacement.[11]

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Presented at the 27th Annual Scientific Meeting of the American Urogynecologic Society, Palm Springs, CA, Oct. 19-21, 2006.

Received July 6, 2006; revised Dec. 29, 2006; accepted Feb. 26, 2007.

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This study was supported by NIH grant HD 38655, the Mary Jane Noble Foundation, the Baden Family Center, and the Scott, Sherwood and Brindley Foundation.

0002-9378/$32.00

doi: 10.1016/j.ajog.2007.02.037

Because performing properly designed experimental studies in women is challenging because of ethical constraints, we have evaluated the squirrel monkey as an animal model of vaginal birth–associated prolapse and have characterized pelvic floor innervation in this model.[13-17] The current study was performed to determine whether evidence of myogenic or neurogenic muscle injury exists in the LA (pubocaudalis and iliocaudalis muscles) of female squirrel monkeys with and without POP. In addition, histopathology was examined in the paravaginal attachments, which are bilateral condensations of connective tissue over the pubocaudalis muscle providing lateral vaginal support, similar to the arcus tendineus fasciae pelvis in women. Disruption of these attachments occurs in women and is associated with anterior vaginal wall descent.[18]

Materials and Methods

Animal subjects

Animals were obtained from the University of South Alabama Primate Research Laboratory and were housed at the Scott & White Hospital Animal Facility in Temple, TX. A total of 19 female squirrel monkeys were used ranging in age from 4 to 25 years, including 6 parous females with POP, 7 parous females without POP similar in age, parity, and weight to females with POP, and 4 nulliparous females without POP (including 3 females ≤6 years old and 1 that was 23 years old). All female squirrels with POP had anterior and/or posterior segment defects that extended beyond the vaginal opening by using a previously reported pelvic examination procedure.[13] For comparison, muscles from 2 females that underwent bilateral LA nerve transection[15] 2.5 years before collection were also evaluated. Twelve of the monkeys used in this study were also used as part of a previous study to examine LA muscle volume in vivo using magnetic resonance imaging (MRI).[17] Guidelines for the care and use of these animals, approved by The Scott & White Institutional Animal Care and Use Committee, were followed.

Necropsy and tissue collection

Tissues from all animals were collected during the nonbreeding, nonovulatory season when estrogen levels are lowest to eliminate histologic differences that could be related to hormone status.[13,14] Monkeys were preanesthetized with 25 mg ketamine intramuscularly (IM) (RXVeterinary Products, Grapevine, TX) and deeply anesthetized with 750 mg sodium pentobarbital IP (Henry Schein, Inc, Port Washington, NY). Animals were transcardially perfused with 2 L phosphate buffered saline (PBS). At necropsy, animals were inspected for gross disruption of the pelvic floor musculature, connective tissue attachments, and innervation. LA and pudendal nerves, LA (pubocaudalis and iliocaudalis) muscles, and pelvic viscera were removed from each animal as described previously.[15] Careful dissection of the vagina from the LA muscle using a dissection microscope exposed the paravaginal attachments (PVA) as bilateral dense connective tissue bands adjacent to the pubocaudalis muscle. Thus, the pubocaudalis muscle and attached PVA were processed for histology simultaneously. Left and right pubocaudalis and iliocaudalis muscles were weighed, fixed 2 hours in 4% paraformaldehyde, cryoprotected in sucrose solutions, and frozen as described previously.[15] Tissue blocks were stored at −70°C until sectioning.

Determination of myocyte size

Serial transverse sections were cut at −20°C (12 μm thick) and collected onto Kling-On HIER slides (Biocare Medical, Walnut Creek, CA). Two to four sections per muscle were stained with wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate (WGA-TRITC; Sigma, St. Louis, MO) as described previously[15] to label myocyte surfaces and thereby enable the determination of myocyte size. Digital images were obtained by using an Olympus Provis AX-70 epifluorescence microscope with appropriate filters (Olympus America Inc, Melville, NY) and a cooled charge-coupled device camera (Olympus America Inc). The best quality tissue section for each muscle was chosen for analysis, and 9 to 15 fields (400×) that were distributed throughout the section in similar regions for each muscle were photographed by an author (S. B. or M. R.) who was blinded to the POP status, parity, and age of each animal. Diameters were then measured in all myocytes that were contained in each image, which resulted in the analysis of at least 100 myocytes per muscle (range, 112-235 myocytes). Cross-sectioned myocyte profiles were outlined with the SigmaScan Pro 5.0 (SPSS Inc, Chicago, IL) software package, and the length of the minor axis of the best-fit ellipse was determined automatically for each profile. We used this length measurement as an estimator of myocyte "least diameter," which is a preferred indicator of myocyte size because it compensates for oblique section angles.[12]

Variation in fiber diameter was defined as a variation coefficient of greater than 30%.[5,6,12] Fiber atrophy was defined as a myocyte having a diameter less than 50% of the control group,[5,6] whereas hypertrophy was defined as a myocyte exceeding twice the diameter of the control group.[4-11] The control group in this study consisted of nulliparous young adult monkeys 4 to 6 years of age (n = 3).

Fiber typing

Additional sections were fixed 10 minutes in acetone, rinsed 10 minutes in water, washed 3 × 5 minutes in 0.2% TPBS (Triton X-100/PBS), and preincubated 1 hour in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 0.3% TPBS. Sections were incubated overnight at room temperature with either a monoclonal antifast skeletal myosin antibody (clone MY-32; Sigma) or a monoclonal antislow skeletal myosin antibody (clone NOQ7.5.4D; Sigma) each at a dilution of 1:100 (2-4 sections per muscle were stained with each antibody). After washing 3 to 5 minutes in 0.2% TPBS, sections were incubated for 2 hours with a fluorescein isothiocyanate-conjugated horse antimouse secondary antibody (1:100