OBSTETRICS

The persistence of active smooth muscle in the female rat cervix through pregnancy

David J. Ferland, BA; Emma S. Darios, BS; Stephanie W. Watts, PhD

OBJECTIVE: A controversy exists as to whether functional smooth muscle exists in the cervix before and during pregnancy, potentially continuous with the uterus. We hypothesized that cervical smooth muscle persists through pregnancy and is functional.

STUDY DESIGN: Uteri and cervices were taken from female virgin, 11 day, and 20 day (near labor) pregnant rats. All experiments used the uterus as a positive control. Three different smooth muscle proteins (smooth muscle α-actin, SM-22α, and calponin-1) allowed immunohistochemical detection of the continuous nature of the smooth muscle from the vagina, cervix, and uterus. Tissues were also hung in isolated tissue baths for the measurement of isometric smooth muscle contraction. Uterine and cervical homogenates were also used in Western analyses to measure protein expression.

RESULTS: Immunohistochemistry revealed there to be smooth muscle as validated by an expression of all 3 markers in the cervix. This smooth muscle was continuous with that of the vagina and uterus. Smooth muscle α-actin was detected in virgin tissue (291.3 $\pm$ 32.2 arbitrary densitometry units/β-actin), day 11 (416.8 $\pm$ 19.5), and day 20 pregnant tissue (293.0 $\pm$ 34.4). The virgin, day 11, and day 20 cervices contracted 2.18 $\pm$ 0.24 g, 1.46 $\pm$ 0.08 g, and 3.88 $\pm$ 0.49 g (respectively) to depolarizing KCl. Cervices contracted at day 20 to the cholinergic muscarinic agonist carbamylcholine (maximum, 133% $\pm$ 18.2% KCl contraction, n = 4).

CONCLUSION: These findings strongly support that smooth muscle is present in the cervix through pregnancy and continuous with the uterus.

Key words: cervix, pregnancy, rat, smooth muscle

Cite this article as: Ferland DJ, Darios ES, Watts SW. The persistence of active smooth muscle in the female rat cervix through pregnancy. Am J Obstet Gynecol 2015;212:244.e1-8.

The existence and role of smooth muscle in the cervix has been debated for decades. The issue was first introduced in the late 19th century when collagen and muscle were discovered in the cervix.[1] The muscular attributes of the cervix were supported in 1934 when experiments were done to test the ability of the separated uterus and cervix to contract. Strips of a goat cervix contracted to adrenaline and acetylcholine but not to oxytocin.[2] By 1947, based on the writings of Danforth,[3] it can be inferred that the field had been nearly convinced of the cervix as an active, muscular organ. However, Masson and Milligan trichrome staining supported that the cervix lost smooth muscle in pregnancy, becoming primarily collagenous.

Histological evidence furthers the case for a collagenous passive cervix and structural remodeling,[4,5] but isolated tissue bath experiments support the case for a muscular and active cervix.[6-12] The inability to come to an agreeable conclusion hinders our ability to effectively investigate pressing issues surrounding pregnancy, such as premature labor.

One of the fundamental problems when studying the uterus and the cervix as separate entities is defining a microscopic histological boundary between the two. Because of the ambiguous nature of such a boundary, the proper sampling of cross-sectional imaging and immunohistochemistry is difficult to accomplish. In this study, we used methods not previously used and interrogated whole tissue with an array of smooth muscle markers. By removing the uterus, cervix, and vaginal tissue as 1 unit and viewing the tissue along the long axis of the lumen, we could examine the smooth muscle of the vagina, cervix, and uterus in 1 picture.

Based on macroscopic cues like changes in color and texture, we were able to separate the tissue of the uterus from that of the cervix and conduct a proof of concept experiment to measure the smooth muscle of the cervix quantitatively via Western blots. The presence of a measureable amount of smooth muscle as identified by smooth muscle α-actin, calponin-1, and SM-22α in the cervix at day 20 of a 21 day gestation period is sufficient proof of the hypothesis that smooth muscle persists through pregnancy. By this method of dissection, we were also able to perform isometric contraction assays and test the activity of this smooth muscle in a near-labor tissue, examining the hypothesis that

From the Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI.

Received April 23, 2014; revised June 13, 2014; accepted Aug. 4, 2014.

This study was supported by a Collaborative Clinical and Translational Research grant (S.W.W.)

The authors report no conflict of interest.

Presented at the Second Annual MSU Conference on Women's Health Research, East Lansing, MI, Nov. 22, 2013.

Corresponding author: Stephanie W. Watts, PhD. wattss@msu.edu

0002-9378/$36.00 • http://dx.doi.org/10.1016/j.ajog.2014.08.001

it will remain functional throughout pregnancy.

Materials and Methods

Animal care and breeding

All animals were used and treated in accordance with protocols approved by the Michigan State University Institutional Animal Use and Care Committee. Virgin female Sprague-Dawley rats (~225 g; Charles River Breeding Laboratories, Portage, MI) were used. To breed, virgin females were placed into wire cages with male Sprague-Dawley rats (varied ages and weights; Charles River Breeding Laboratories, Portage, MI) for several days (1 male to 1 female). The presence of a vaginal plug was assumed to indicate conception and recorded as day 1 of pregnancy. Females were separated into individual cages and euthanized (via isoflurane and pneumothorax) at a specific time after conception plus or minus1 day of the target periods (day 11 and day 20 of a 21 day gestation period).

The ovaries, bilateral uterine horns, cervix, and vaginal tissue were removed (Appendix; Supplemental Figure) and placed into a physiological salt solution: 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_3, 1.17 mM $MgSO_4 \cdot 7H_2O$, 1.16 mM $CaCl_2 \cdot 2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, and 0.03 mM $CaNa_2 \cdot$ EDTA (pH 7.2). The results described in the following text use the cervix and uterus that were separated from the same rat.

Histology and immunofluorescence

Tissues were cleaned of fat and the vaginal tissue remained connected to the cervix and uterus. An incision was made longitudinally that allowed the uterus/cervix to lie open and flat. The tissue was pinned flat tautly and fixed with 10% formalin for 24-48 hours. Tissues were then transferred to 70% ethanol. The Michigan State University Investigative HistoPathology laboratory paraffin embedded, sectioned, and prepared Masson trichrome slides of this tissue as well as blanks.

Paraffin-embedded slides were cleared, unmasked for 1 minute (citrate-based antigen unmasking solution; Vector Laboratories, Burlingame, CA), blocked, and incubated for 1 hour at 37°C with fluorescein isothiocyanate (FITC)-conjugated anti—smooth muscle α-actin (5 μg/mL; Sigma-Aldrich, St. Louis, MO) or with a primary antibody (anti-SM-22α, 1:200; Abcam, Cambridge, MA; anticalponin 1, 1.5 μg/mL; Thermo Scientific, Rockford, IL) followed by 30 minutes at 37°C with secondary antibody (Alexa Fluor 488, 1:1000, 1:500, respectively; Invitrogen, Eugene, OR). Negative controls were sections without the primary antibody. Prolong Gold with 4′,6-diamino-2-phenylindole (DAPI; Invitrogen) was added to all tissues before applying coverslips. Slides were photographed using a Nikon (Tokyo, Japan) Eclipse Ti inverted microscope at a ×4 magnification with NIS-Elements software (Nikon).

Image analysis

Each photograph is a combination of a DAPI, FITC, and tetramethylrhodamine isothiocyanate (TRITC; blank) channel and standardized for true fluorescence based on the control TRITC taken for that specific photograph that was then embedded into the photograph and carried through all image analysis. Both exposure times and look up table adjustments were made based on the TRITC autofluorescence. These overlays were sealed together and stitched using Adobe Photoshop C6 (Adobe, San Jose, CA). Because of the slight differences in each photo, the brightness and contrast were adjusted to maintain continuity through the stitching process. The carrying of the TRITC channel through these manipulations ensured that no colors were misrepresented.

Protein isolation and Western blots

Tissues were cleaned of fat, vaginal tissue, and ovaries. The cervix and uterus were split based on a previously described visual/textual border.[12] Additional tissue was removed from each end of both sections to ensure that no uterine tissue remained on the cervical section and vice versa. Both cervices were also left together and not separated. If placentas and embryos were present, they were also removed. The tissue was homogenized and analyzed for total protein concentration using a bicinchoninic acid assay (BCA1-1KT; Sigma-Aldrich). Fifty micrograms of protein were loaded and run into a 12% polymerized acrylamide.

Proteins were transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare, Piscataway, NJ) and blocked with 4% chick egg ovalbumin (Sigma-Aldrich) for 3-24 hours at 4°C. Incubation with primary antibodies occurred overnight at 4°C (anti-β-actin goat, 1:500; Abcam; anti—smooth muscle-specific α-actin mouse, 1:2000; Calbiochem, Darmstadt, Germany; anti-SM-22α rabbit, 1:2000; Abcam; anti-calponin-1 mouse, 1:2000; Thermo Scientific). Blots were visualized on an Odyssey LI-COR FC (LI-COR, Lincoln, NE) with Odyssey LI-COR IRDye secondaries (antigoat 680LT, 1:1000; antimouse 800CW, 1:1000; antirabbit 800CW, 1:1000; antimouse 800CW, 1:1000, respectively) incubated for 1 hour at 4°C. Image quantification was performed using densitometry in ImageJ (version 1.46r; National Institutes of Health, Bethesda, MD). Data are reported normalized to β-actin.

Isolated tissue bath contractility

The uterus and cervix were cleaned and divided as described in previous text. The cervix with a total length of 1 cm was hung on L-shaped hooks (1 hook through each lumen). The uterus was cut longitudinally and trimmed to a 2 cm × 1 cm long strip. Although the size of each tissue was not strictly used to normalization, it was consistent throughout. Tissues were placed into 50 mL baths with physiological salt solution, warmed to 37°C, and aerated with 95% O_2/5% CO_2. Force of contraction was captured by an ADInstruments Inc (Colorado Springs, CO) PowerLab (model 4) and Quad bridge along with Lab Chart 7 on an MSi computer.

Once the tissues were connected to the transducers (FT03; Grass Instruments, Quincy, MA), approximately 1 g of passive tension was pulled to bring the smooth muscle to its optimal length, and the baths were washed every 10 minutes for 1 hour. The tissues were first