Myoarchitectural and functional alterations in rabbit external anal sphincter muscle following experimental surgical trauma

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Rajasekaran MR, Sinha S, Seo Y, Salehi M, Bhargava V, Mittal RK. Myoarchitectural and functional alterations in rabbit external anal sphincter muscle following experimental surgical trauma. Am J Physiol Gastrointest Liver Physiol 307: G445–G451, 2014. First published July 3, 2014; doi:10.1152/ajpgi.00450.2013.—Obstetrical trauma to external anal sphincter (EAS) is extremely common; however, its role in the development of anal incontinence is not clear. We examined the regenerative process and functional impact of experimental surgical trauma to EAS muscle in an animal model. Surgical myotomy, a craniocaudal incision extending along the entire length and thickness of the EAS, was performed in rabbits. Animals were allowed to recover, and anal pressures were recorded at weekly intervals for 12 wk using a custom-designed probe system to determine the length-tension property of EAS muscle. Animals were killed at predetermined time intervals, and the anal canal was harvested for histochemical studies (for determination of muscle/connective tissue/collagen) and sarcomere length measurement. In addition, magnetic resonance diffusion tensor imaging (MR-DTI) and fiber tracking was performed to determine myoarchitectural changes in the EAS. Myotomy of the EAS muscle resulted in significant impairment of its length-tension property that showed only partial recovery during the 12-wk study period. Histology revealed marked increase in the fibrosis (connective tissue = 69% following myotomy vs. 28% in controls) at 3 wk, which persisted at 12 wk. Immunostaining studies confirmed deposition of collagen in the fibrotic tissue. There was no change in the sarcomere length following myotomy. MR-DTI studies revealed disorganized muscle fiber orientation in the regenerating muscle. We conclude that, following experimental injury, the EAS muscle heals with an increase in the collagen content and loss of normal myoarchitecture, which we suspect is the cause of impaired EAS function.

Of the three components of the anal sphincter complex [the internal anal sphincter, external anal sphincter (EAS), and puborectalis muscle (PRM)], it is generally believed that EAS muscle plays a significant role in the development of FI (2, 30). Functional data, measured as anal canal pressure, show lower rest and squeeze anal canal pressures in FI patients compared with controls (23). A recent study from our laboratory reveals that the length-tension property of the EAS and PRM is significantly impaired in FI patients (14). Furthermore, length-tension dysfunction correlates with the severity of defects in the EAS and PRM as seen on ultrasound (US) imaging. Injury to the muscle is an important cause of impairment in its length-tension function; for example, injury to myocardium and limb muscles impairs their length-tension property (9, 12, 18). Lower esophageal sphincter function, as assessed by its length-tension measurement, is impaired in patients with gastroesophageal reflux disease (5).

Several investigations have examined the effects of EAS injury on anal canal function and mechanisms of repair (27–29, 34). In addition, beneficial effects of direct injection of stem cells in injured anal sphincter at the time of the repair has also been explored (17). However, most of these studies were short-term and employed in vitro parameters of muscle function. To the best of our knowledge, there are no studies on the effects of trauma to the EAS muscle on its length-tension property in vivo. Furthermore, the time course of formation of fibrosis and alterations to the EAS myoarchitecture is not clear. Therefore, our goal was to conduct a longitudinal study of the effects of EAS muscle injury on various aspects of anal sphincter morphology and function, in vivo, in an animal model.

MATERIALS AND METHODS

The institutional animal care and use committee at the Veterans Affairs San Diego Healthcare Systems approved the study protocol, and all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Adult New Zealand White female rabbits (n = 13; 4–5 kg) were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). An intravenous catheter was placed (ear vein) for maintenance of anesthesia and administration of drugs.

Two custom-designed copper-wire hook electrodes were placed in the EAS muscle for electrical stimulation, one at the 3:00 position and the other at the 9:00 position. The anal canal pressure was measured using manometric methods; a 3-mm-diameter sleeve sensor was placed in custom-designed probe holders of 4.5, 6, and 9 mm diameter (Fig. 1A). The anal canal pressures were recorded with the pressure-sensing surface of the sleeve sensor facing the posterior midline direction (19, 21, 22). A pulse generator (model S48; Grass Technol-
Three animals were killed at week 3 and another three at week 12 after the myotomy. The anal canal was harvested and fixed in formalin to perform the following: 1) histology and light microscopy to determine fibrosis and to quantify muscle/connective tissue, 2) sarcomere length by laser diffraction method to evaluate muscle sarcomere length, and 3) magnetic resonance imaging (MRI) to determine myofiber orientation and changes after injury.

**Histological evaluation.** The skin was removed, and anal canal sections were processed. Paraffin tissue sections (7 μm) were applied to the microscopic slides, deparaffinized, and stained with Masson trichrome (muscle/connective tissue) and Sirius red (for collagen). Digital images of the entire anal canal cross section (muscle and mucosa) were captured. The image analysis was performed to quantify connective tissue/collagen and skeletal muscle components (Nikon Elements, Melville, NY) (24). Briefly, the amount of fibrosis formation on the injured and contralateral side was measured by selecting two representative and nonadjacent sections and photographing up to three microscopic fields. Muscle and connective tissue components were identified by red and blue color stains, respectively. Images were quantitated for red pixels (stained for skeletal muscle) and blue pixels (connective tissue components), estimating them as a percentage of the total number of pixels.

**Sarcomere length.** Formalin-fixed anal canal specimens were rinsed in phosphate-buffered saline and placed in 15% sulfuric acid (8–12 h) to partially digest the connective tissues surrounding the muscle. The EAS muscle fibers were isolated by microdissection, mounted on microscopic glass slides, and subjected to laser diffraction for determination of the sarcomere length as described earlier (16, 19, 21, 22). The muscle fiber bundle was transilluminated by a He-Ne laser (model 05-LHR-171; Melles-Griot, Irvine, CA), and the sarcomere length was calculated via the grating equation: 

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\text{Sarcomere length} = \frac{n \lambda}{d \sin \theta}
\]

where \(n\) is the diffraction order (± 1, ± 2, ± 3, etc.), \(\lambda\) is the laser wavelength (0.632 μm), \(d\) is the grating spacing (which equals sarcomere length), and \(\theta\) is the diffraction angle measured by use of a photodiode array (16).

**High-resolution MRI, diffusion tensor imaging, and fiber tracking studies.** To determine the myoarchitectural changes of the EAS muscle, MRI and diffusion tensor imaging (DTI) were performed on all eight specimens in a 7T scanner (7T Bruker Avance II Biobspin system; Bruker Medical Systems, Germany). A high-resolution MRI was obtained with a two-dimensional RARE double-echo sequence with echo time 1 \(T1\)/2E/repetition time \(T2\) of 17/52/5,000 ms, slice thickness: 0.5 mm, contiguous; matrix: 256 × 256; field of view (FOV): 3 × 3 cm; in a scan time of ~22 min. With the use of the same slice positions, a DTI of the sphincter muscle was carried out with a single-shot spin-echo sequence \(T2/T2\) of 26/18,750 ms with a b value of 600 s/mm², 32 diffusion gradient directions, and 5 baseline images. Geometry parameters included: 3 × 3 cm FOV, 256 × 256 matrix, 0.5 mm thick/0.5 mm gap, and ~70 slices to provide coverage of the entire specimen to match the high-resolution images. The Bruker format images were then analyzed using DTI Studio to calculate the diffusion tensor, which yielded the fractional anisotropy (FA), and the three eigenvectors for the control and myotomy specimens. The tensor images were then used for fiber tracking and visualization, using typically an FA threshold of 0.15, minimum/maximum of 4 and 40-mm fiber length, and 50° maximum turning angle/step. The methods of analysis of DT images used here are described in detail in our previous publication (25). These tensor images and fiber tracking for different slice positions in the control and myotomy specimens were compared with histological images of the corresponding slices.

**Data analysis.** All pressures were measured in reference to atmospheric pressure. Rest pressure was determined as the average pressure recorded 30 s before the EAS muscle stimulation. The maximum pressure during EAS stimulation was the peak pressure recorded during the 10-s stimulation period. Delta pressure was defined as the difference between the maximum and rest pressure. The force of contraction of the EAS muscle was determined by calculating muscle...
tension (T_m) using the following equation: \( T_m = P \times r_m/t_m \), where \( P \) is the intraluminal pressure, \( r_m \) is the inner radius of the EAS muscle, and \( t_m \) is the EAS muscle thickness. The \( r_m \) and \( t_m \) were measured from the US images (using a 2-mm-diameter US catheter) of the rabbit’s anal canal and further confirmed by measuring these parameters in a freshly harvested specimen. EAS muscle thickness was calculated mathematically for each probe size, assuming conservation of mass. The EAS muscle tension represents the average circumferential force per unit area of the circular muscle and is denoted in milli-Newton per square centimeter (21).

Statistical analysis. Data are shown as means ± SE. For length-tension studies, a two-way repeated-measure ANOVA with post hoc Tukey’s test (SPSS) was performed. For muscle/connective tissue and sarcomere length evaluations, one-way ANOVA followed by Bonferroni post hoc test was employed.

RESULTS

Effect of the EAS surgical myotomy on the anal canal function. Effects of the EAS myotomy on the anal canal pressure and EAS muscle tension are summarized in Fig. 2. Representative tracings demonstrate the effect of EAS myotomy and electrical stimulation on the anal canal pressure. Before myotomy, maximal (6-mA) electrical stimulation of the EAS muscle produced a probe-dependent increase in the anal canal pressure (Fig. 2A). EAS myotomy resulted in 80–90% reduction in the anal pressures in response to an identical stimulation before the myotomy. Anal canal pressure with the largest-diameter probe (9 mm) dropped from 312 mmHg before myotomy to ~27 mmHg immediately after myotomy and then gradually returned to ~104 mm at the end of week 4 postmyotomy. Despite some recovery, the phenomenon of probe-dependent increase in the anal canal pressure and length-tension function remained impaired throughout the study period.

Length-tension property of the EAS muscle before, after injury, and during the recovery process is summarized in Fig. 2B. Before myotomy, maximal electrical stimulation of the EAS produced a steep, probe-dependent increase in the muscle tension (red line) that became relatively flat immediately after myotomy (purple line). The muscle tensions (mN/cm²) recorded before injury for 3-, 4.5-, 6-, and 9-mm probes were 787 ± 99, 1,123 ± 120, 1,658 ± 118, and 2,688 ± 173, respectively. At 4 and 12 wk following myotomy, the EAS muscle tension developed in response to maximal electrical stimulation was significantly reduced compared with the premyotomy values. At 4 wk, EAS muscle tension was significantly smaller with all probe sizes (445 ± 107, 504 ± 65, 602 ± 74, and 945 ± 153, respectively, for 3, 4.5, 6, and 9 mm) compared with before myotomy. At 12 wk postinjury, a significant difference in the muscle tension (mN/cm²) was noted with 6- and 9-mm probes (944 ± 205 and 1,630 ± 405, respectively).

Histology. Figure 3, A–D, shows representative photomicrographs of the anal canal cross-sectional anatomy stained with trichrome and Sirius red, before and after injury. In trichrome-stained sections, the red color represents muscle and blue is the connective tissue. In Sirius red-stained sections, the red color indicates collagen. Before injury, the anal canal cross section shows that the EAS muscle fibers are arranged in a uniform pattern with a relatively small amount of connective tissue (Fig. 3A). One week after myotomy, a marked increase in the connective tissue was noted in the specimens (Fig. 3B). Connective tissue deposits were seen in the injured area and the noninjured area of the EAS (Fig. 3B). Sirius red staining confirmed that the increased connective tissue was due to an increase in the collagen deposits, both at the injury and the noninjury sites. At 3 wk postmyotomy, there was progressive increase of fibrosis that was widespread and persisted at 12 wk. Image analysis of the trichrome stain anal canal sections revealed a significant increase in the connective tissue content after EAS myotomy. In control, the uninjured sphincter, the muscle and connective tissue contents were 72 ± 6 and 28 ± 0%, respectively. At 3 wk postmyotomy, the muscle-to-connective tissue ratio was reversed (muscle 31 ± 1% and connective tissue 69 ± 1), and these changes persisted at week 12 (P < 0.05; Fig. 4, top) on the injured side. The connective tissue contents (%) on the contralateral side at 3 and 12 wk postinjury were 46 ± 5 and 53 ± 7% (P < 0.05), respectively.

Sarcomere length. Figure 4, bottom, shows EAS sarcomere length in microns (µ) before myotomy and 12 wk after myotomy. Sarcomere length before and 12 wk postmyotomy was 2.03 and 2.1 µm, respectively; the difference was not significant.

MRI-DTI studies. Representative photomicrographs of trichrome-stained anal canal cross sections are shown in Fig. 5, A (normal) and B (12 wk postmyotomy). The site of surgical incision (myotomy) was clearly visualized in the histological image (Fig. 5B) and in the magnetic resonance (MR) images (Fig.
We used trichrome-stained images to validate and corroborate EAS anatomy obtained from MR studies in the different specimens. The histological images compared very well with the proton-density MR images at identical slice locations shown in Fig. 5, C (normal) and D (injured). No quantitative analysis of DTI images was performed. Visual examination of the diffusion tensor (λ1) images shows a homogenous distribution in the normal EAS (Fig. 5E) and heterogeneous appearance in the injured muscle (Fig. 5F). The red color in these images represents anterior/posterior direction, the green represents left/right direction, and the blue color represents the craniocaudal direction of muscle fibers with respect to the magnet axis. The “speckled” appearance of the tensor values, we suspect, is “most likely” indicative of the presence of fibrosis. DTI fiber tracking based on the tensor images shown in Fig. 5, E and F, reinforced the presence of continuous intact fibers that follows circular trajectory in the normal EAS (Fig. 5G). Figure 5I shows a side view of the circular fibers of the EAS; it reveals the continuous regular circular structure of the normal EAS.

**DISCUSSION**

The goals of our studies were to evaluate the time course of spontaneous healing of the EAS sphincter muscle following a controlled surgical myotomy. We evaluated EAS muscle function by determining its length-tension property, fibrosis, and collagen deposits in the muscle and changes in the myoarchitecture using a relatively novel noninvasive MR-DTI technique. We chose the rabbit model based on our extensive experience and a recent report supporting its use for coloproctology research (10). Overall, our results show that a surgical myotomy of the EAS results in the following: 1) the loss of muscle function, i.e., alteration in its length-tension property; 2) excessive collagen deposition and reduction in the muscle sarcomere length (L0).

**Fig. 3.** Masson trichrome and Sirius red stain of rabbit EAS from controls (A) and 1 (B), 3 (C), and 12 (D) wk post-myotomy. Histological evidence of sustained and widespread fibrosis is demonstrated with Masson trichrome and Sirius red stain. The EAS muscle is stained in red and connective tissue in blue in these images. Collagen is red in color with Sirius red stain.

**Fig. 4.** A: effect of EAS myotomy on the muscle and connective tissue. B: EAS muscle sarcomere length before and after myotomy (n = 3 at each time point).
mass based on histology, and 3) myoarchitectural disarray of EAS muscle due to the loss of muscle fiber directions (based on MR-DTI).

Skeletal muscle fibrosis is defined as an abnormal, unresolvable, and chronic increase in the extracellular connective tissue that interferes with the organ function (15). Fibrosis is the final common pathological outcome of many chronic inflammatory diseases (33). Normal tissue repair can evolve into progressively irreversible fibrotic response if the tissue injury is severe or repetitive, or if the wound-healing response itself is dysregulated. Persistent fibrosis can lead to permanent scarring and organ malfunction (33). Increased collagen and defective collagen cross-link has been shown to impair myocardial length-tension function (6). Trauma to leg muscle in rats also causes deterioration in its length-tension function. In our study, EAS muscle injury resulted in progressive extensive fibrosis on both the injured and on the contralateral side; to the best of our knowledge, this is a novel finding. We used the conventional histological/histochemical approach (Masson’s trichrome for extracellular matrix and Sirius red for collagen) to evaluate fibrosis/collagen in the EAS muscle; it is a well-established technique to study fibrosis/collagen in many organ systems, including the skeletal muscles (15, 33).

Besides the histochemical approach, we used MR-DTI and DTI-based Fiber Tracking technique to confirm myoarchitectural disarray after EAS injury. Previous studies in the skeletal muscle injury experiments tested the hypothesis that soft tissue such as muscle tissue is ordered when healthy and disordered when injured (32). Studies of cardiac and other skeletal muscles (7, 31, 32) indicate that DTI can detect tissue organization and disorganization in healthy and diseased muscles. Furthermore, DTI can detect changes in the myocardial structure at the cellular levels (7, 31), and these changes correlate with functional impairment. Visual examination of the EAS muscle DTI shows a speckled appearance in the EAS muscle region. Speckled appearance refers to a large degree of noise in the diffuse tensor images and most likely indicates myofibers interspersed with fibrotic content. Fibrotic tissue contains a high degree of collagenous material that would be expected to manifest as a signal void in the MR image. We speculate that fibrous tissue when admixed with a certain amount of normal muscle shows a speckled appearance on the diffusion tensor images, as seen in Fig. 5F. Visualization of the muscle fiber tracks from the DT images revealed random orientation and a disordered state of sphincter muscle fibers in the injured muscle. Despite the novelty, there are several limitations with our DTI and fiber tracking techniques: a detailed quantitative DTI analysis and reproducibility of the analysis were not performed. In addition, direct correlations of DTI quantitative analysis with muscle histology parameters were also not attempted. Therefore, one may consider our MRI findings as preliminary and require further validation. We believe though that MR-DTI is a powerful tool because it allows one to assess myoarchitecture and fibrosis in a noninvasive manner, in contrast to muscle histology.

Overall our histological and MRI studies confirm fibrosis: excessive collagen deposits leading to disordered fiber arrangements in the regenerated muscle. Alteration in the length-tension property of the EAS muscle or loss of muscle function in our study is explained by reduction in muscle mass, increase in collagen, increases in fibrosis, and loss of myoarchitecture of the EAS muscle rather than the change in sarcomere length. We expected that, with time, there would have been regeneration of muscle and complete restoration of length-tension function of the EAS muscle; however, it was not detected during the 3-mo study period. For length-tension evaluations, muscle thickness was measured with a 2-mm US probe. Applying the law of conservation of mass, this measurement was used to estimate muscle thickness for 4.5-, 6-, and 9-mm probes. We believe these estimates are accurate. It is possible that, with a longer observation period, there may have been greater recovery of the sphincter function. Based on the published studies, however, muscle regeneration is usually complete by one month (8).

Anal sphincter damage during childbirth is common. The degree of damage (1st-, 2nd-, and 3rd-degree tears) is well

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Fig. 5. Representative rabbit anal canal cross sections showing EAS histology (A and B), magnetic resonance proton density (C and D), diffusion tensor imaging (DTI, E and F), and fiber directions (G–I) showing myoarchitectural changes and fiber directions. Top, control; bottom, after injury as described in MATERIALS AND METHODS.
recognized soon after parturition, but the occult injuries to the EAS and PRM are underappreciated. US imaging reveals defects of EAS in 8–25% of women after childbirth (26). Along the same lines, three-dimensional US imaging and MRI reveals defects of PRM in 25–35% of the parous women (14). The significance of occult injuries to the EAS and PRM in the development of FI is not clear. Also not clear is the late onset of FI symptoms in women because obstetrical trauma occurs when women are in their 20s and 30s (childbearing years) but symptoms do not develop until they are 50–60 yr old. Recently, we found that the length-tension of the EAS and PRM is severely impaired in women with FI, and there is correlation between the US-detected defects and loss of EAS and PRM function (14). It may be that the age-related deterioration in the muscle function adds to the loss of function associated with occult damage, and the two together lead to a critical low-level function required for development of FI symptoms. It is also possible that damaged muscle deteriorates with aging much more rapidly than normal muscle. One of the limitations of our study is that we used a standardized surgical incision to induce EAS trauma. On the other hand, obstetrical trauma to the sphincter muscles may occur through several different mechanisms (stretch, avulsions, instrumentation, and episiotomy; see Ref. 11). In addition, the controlled experimental injury in our animal model may be more severe than the obstetrical injury. We could not measure rate of force development during EAS contraction to evaluate the possible impact of fiber-type changes in response to injury because the sleeve sensor that we used for pressure measurement has slow response rate and may not capture the rate of change of pressure accurately.

In conclusion, surgical trauma to the EAS muscle leads to significant alterations in its structure and function. Future studies should focus on novel interventional strategies to attenuate fibrosis and enhance normal muscle regeneration following obstetrical and other types of trauma to the EAS and other pelvic floor muscles.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: M.R.R., V.B., and R.K.M. conception and design of research; M.R.R., S.S., Y.S., and M.S. performed experiments; M.R.R., S.S., and R.K.M. wrote the initial draft of the paper; and by a limited project grant from ASCRS research foundation.

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