Age Related External Anal Sphincter Muscle Dysfunction & Fibrosis: Possible Role of
Wnt-β Catenin Signaling Pathways

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ABSTRACT:

Studies show an age-related increase in the prevalence of anal incontinence and sphincter muscle atrophy. Wnt-β catenin signaling pathway has been recently recognized as the major molecular pathway involved in the age-related skeletal muscle atrophy and fibrosis. The goals of our study were to evaluate: (i) impact of normal aging on the EAS muscle length-tension (L-T) function & morphology; and (ii) specifically examine the role of Wnt signaling pathways in anal sphincter muscle fibrosis. New Zealand White female rabbits (six young 6 months old and six old 36 months of age were anesthetized anal canal pressure was measured to determine the L-T function of EAS. Animals were sacrificed at the end of study and anal canal was harvested and processed for histochemical studies (Masson trichrome stain for muscle/connective tissue) as well as for molecular markers for fibrosis and atrophy (collagen-I, β-catenin, TGF-β, atrogin-1 and MURF-1). The L-T was significantly impaired in older animals compared to young animals. Anal canal sections stained with trichrome showed a significant decrease in the muscle content (52% in old compared to 70% in young) and an increase in the connective tissue/collagen content in the old animals. An increased protein and mRNA expression of all the fibrosis markers were seen in the older animals. Aging EAS muscle exhibits impairment of function and increase in connective tissue. Up regulation of atrophy and pro-fibrogenic proteins with aging may be the reason for the age-related decrease in anal sphincter muscle thickness and its function.
**New & Noteworthy:** Our studies using a female rabbit model show age-related alterations in the structure and function of the EAS muscle. We used endoluminal ultrasound to measure age-related changes in EAS muscle thickness. We employed Western blot and quantitative PCR to demonstrate age-related changes in the levels of important fibrogenic as well as atrophy markers. Our findings may have significant clinical implications, i.e., use of specific antagonists to prevent age-related EAS muscle dysfunction.
Fecal incontinence (FI) is defined as the inability to control bowel movements, causing accidental, unintentional loss of solid or liquid stool from the rectum. It is extremely common, known to have devastating effect on the quality of life and is a cause for institutionalization in the elderly. The negative impact of FI on the woman’s physical and emotional health is tremendous including reluctance to consider future pregnancies (5, 38).

Physiological aging is recognized to be a significant risk factor for FI. A survey by Whitehead et al confirms a strong association between FI and age, with the prevalence of FI increasing from 2.6% at ages 20–30 years to 15.3% in people > 70 years (37). In addition, child birth related injury to the anal sphincter, another important risk factor for FI in women, was shown to exhibit a cumulative impact on the age-related sphincter dysfunction (8). Several epidemiological studies have shown age-related reduction in the anal resting as well as squeeze pressures (3, 17, 37). These observations implicate the involvement of anal sphincters and suggest that these age-related changes to the anal sphincter muscles may predispose to FI in the elderly subjects. Even though a few studies suggest EAS muscle atrophy/fibrosis as a possible cause (26), the exact pathophysiology of age-related alterations in sphincter muscle morphology/function and the underlying mechanisms are still unclear.

A study from our laboratory in a rabbit model proves that following surgical trauma, the EAS muscle regenerates with increase in fibrosis, which is mediated through a novel Wnt-β-catenin pathway (32). In addition, recent reports implicate aberrant activation of Wnt-β catenin signaling pathway in injury as well as age-related limb skeletal muscle fibrosis which suggest involvement of a common pathophysiology between the two risk factors (injury and aging) (9).
Wnt-β catenin signaling pathway is also a major player in the genesis of fibrosis following injury in several organ systems, i.e., myocardium, lungs, kidney, liver, and skin (4). The Wnt/β-catenin signaling is also known to regulate the expression of an important fibrogenic growth factor (TGF-β) which promotes β-catenin signaling (1, 10, 12). Upregulation of Wnt signaling has also been implicated in the age-related fibrosis and dysfunction in the lower limb skeletal muscles (9). In addition, atrogin-1 and MURF-1 are well-recognized markers of skeletal muscle atrophy (7) and role of these proteins in EAS muscle atrophy is unclear.

The goals of our study were to evaluate: (i) the impact of normal aging on the EAS muscle length-tension (L-T) function and morphology; and (ii) if aging is associated with increase in the molecules known to be associated with age-related fibrosis and atrophy in the external anal sphincter muscle. We achieved the above by determining the age-related alterations in the structure and function of the EAS muscle including the levels of important fibrogenic (β-catenin, Collagen-I, and TGF-β1) as well as atrophy markers (atrogin-1, MURF-1) in a rabbit model.
Materials and Methods:

The institutional animal care and use committee at the VA San Diego Healthcare Systems approved the study protocol and all experiments were conducted in accordance with the Guidelines and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Adult New Zealand white female rabbits (n=12; six young (6 months) and six old (36 months) were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). An IV catheter was placed (ear vein) for maintenance of anesthesia and administration of drugs.

Ultrasound images of anal canal were obtained to determine the anal canal radius and EAS muscle thickness. Ultrasound imaging was performed using a 6F, 20 MHz transducer (EU-M30S; Olympus Optical Co; Tokyo, Japan). The probe was placed inside the lumen and advanced into the rectum and then gradually pulled back into that part of the anal canal where external anal sphincter was visualized. The images were recorded on a videotape and analysis was performed later to calculate the anal canal radius and EAS muscle thickness (29-31).

Two custom designed copper wire hook electrodes were placed in the EAS muscle for electrical stimulation at 3’0 clock and 9’0 clock position. The anal canal pressure was measured using manometric methods; a 3-mm diameter sleeve sensor was placed in the custom designed probe holders of 4.5mm, 6mm and 9 mm diameter. The anal canal pressure was recorded with the pressure-sensing surface of the sleeve sensor facing in the posterior midline direction (29-31). A pulse generator (Model S48, Grass Technologies, West Warwick, RI) connected to a constant current unit (Model CCU1A, Grass Technologies, West Warwick, RI) was used for electrical stimulation of the EAS. Electrical stimulus parameters used were current intensity ranging from 1 to 6 mA, in steps of 1 mA increase, pulse frequency 50 Hz and pulse duration of 0.2 ms. Heart rate was monitored continuously during the entire experiment.
Animals were sacrificed at the end of the study and the anal canal was harvested and fixed in formalin to perform the following tissue analysis: 1) Histology & histochemical evaluation to determine the fibrosis/collagen content(2, 25, 32). 2) immuno-histochemistry/immuno-fluorescence to localize proteins involved in fibrosis/ connective tissue formation. 3) Western blots to quantify relevant proteins. 4) Quantitative PCR to determine changes in the gene expression(32).

**Histological Evaluation**: Skin was removed and anal canal sections were processed. Paraffin tissue sections (7µm) were applied to the microscopic slides, deparaffinized and stained with Masson- trichrome (muscle /connective tissue) and Sirius red stain (for collagen). Digital images of the entire anal canal cross section (muscle & mucosa) were captured and image analysis was performed to quantify connective tissue/collagen and skeletal muscle components (*Nikon NIS Elements, Melville, NY*) (32, 35). The amount of fibrosis formation at the site of surgical incision and contralateral side was measured by selecting two representative nonadjacent sections and photographing up to three microscopic fields. Muscle and connective tissue components were identified by red and blue color stain respectively. Images were quantitated for the red pixels (stained for skeletal muscle) and blue pixels (connective tissue components) as percentage of the total number of pixels using *Nikon NIS Elements software*.

**Immunostaining studies**: We performed immunohistochemistry (IHC) to localize collagen-I, β-catenin and TGF-β. For these studies, paraffin tissue sections were processed for antigen staining. Sections were incubated for 30 min with 5% normal goat/horse serum containing 1% Triton X-100 to block the nonspecific binding sites. These sections were further incubated overnight at 4°C with specific monoclonal antibodies for collagen-I, β-catenin and TGF-β (Abcam), (1:200) dissolved in the PBS containing 1% serum. In one set, tissues were incubated
with normal mouse IgG in the absence of primary antibody, which served as a negative control.

After washing 3 times with PBS, the tissues were further incubated for 2 hours at room temperature with appropriate anti-mouse secondary antibodies and processed using vectastain alkaline phosphatase system (Vector labs, CA) to localize the specific proteins. To localize atrophy markers (atrogin-1, MURF-1), we performed immunofluorescence (IF) studies using specific antibodies. Paraffin tissue sections were processed for antigen retrieval. Sections were incubated for 30 min with 5% normal goat/horse serum containing 1% Triton X-100 to block the nonspecific binding sites. These sections were further incubated overnight at 4°C with specific monoclonal antibodies for atrogin-1, MURF-1 (Abcam), (1:200) dissolved in the PBS containing 1% serum. In one set, tissues were incubated with normal mouse IgG in the absence of primary antibody, which served as a negative control. After three washings, sections were further incubated for 2 hours with appropriate anti-mouse secondary antibodies and conjugated with rhodamine. Incubation was terminated by washing with PBS and the slides were mounted in Gel/Mount. The slides were kept in dark at 4°C and observed under a fluorescent microscope within 24 hours for imaging.

**Western Blot** ([32]): To quantify levels of collagen-I, pro-fibrogenic markers (β-catenin, and TGF-β1) and atrophy markers (atrogin-1, MURF-1), samples were first prepared on ice with non-reducing tris-glycine SDS sample buffer (Novex) and heated on a heat block at 95 degrees C for 10 minutes. When reducing conditions were required, mercaptoethanol (5% v/v) was added to the sample buffer. Denatured samples (10 µg) were loaded onto 4–20% NuPAGE tris-glycine SDS polyacrylamide gels and subjected to electrophoresis in tris-glycine running buffer in the Xcell II Minicell, according to the manufacturer's instructions (Novex Australia Pty Ltd, French's Forest, NSW, Australia). Protein standards were included in each gel: 5 L Precision Plus Dual
Color standards. Proteins were transferred to PVDF (Biorad Immunoblot PVDF membrane) overnight at 25 V. Western blots were probed for these specific proteins as follows, all steps were carried out at room temperature, with gentle shaking. After 60 min in blocking buffer (5% Diploma skim milk in PBS), filters were incubated overnight with the optimal dilutions (1:500) of primary antibodies in blocking buffer. After thorough washing in PBS containing 1% Tween 20 (wash buffer), filters were incubated for 2 h in a 1 in 3000 dilutions of appropriate secondary antibody, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit, rabbit anti-mouse IgG (H + L; Jackson Immunosearch, West Grove, PA, USA) in wash buffer. Blots were developed by the enzyme chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham).

**Quantitative PCR(32):** Total RNA was extracted from young and old EAS rabbit samples (n=3 each) using RNA STAT-60 (trizol), treated with RNase-free DNase to eliminate genomic DNA contamination, and purified with RNeasy Fibrous Tissue Mini Kit (Qiagen). An Eppendorf 5415R centrifuge was used to spin down the sample following each step. The cDNA was synthesized from 4 μg total RNA by reverse transcription reaction using Quantitect Reverse Transcription Kit (Qiagen). The primer pairs used for quantitative RT-PCR analysis of TGF-β1, β-catenin and collagen-I have been previously reported(19, 20, 23)

- **β-catenin -Forward:** 5'-ATG TGG ATT TGG AAC CCA AG-3'; **Reverse:** 5'-CCA AAG GGA GGC TTC CTA GT-3'.
- **Collagen I-Forward:** 5’-TAG GCG TTC CAG TTC GAG TA-3'; **Reverse:** 5'GGT CTT CCG GTG GTC TTG TA-3'; **TGF-β1-Forward:** 5'-ACA TTG ACT TCC GCA AGG AC-3'; **Reverse:** 5'-TAG TAC ACG ATG GGC AGT GG-3'). **GAPDH-Forward-5’-GCA CCG TCA AGG CTG AGA AC-3'; reverse:** 5'-ATG GTG GTG AAG ACG CCA GT-3'. **Atrogin-1, Forward-5’-GCAGCTGAACACATTTCAAG-3';**
reverse: 5'- GCCTCTGCATGATGTTCAGT-3'.

MuRF-1-Forward-5'–TGTGCAGACCATCATCACCC-3';

reverse: 5'-AAAGCCCTGCTCTGTCTTCC-3'. Specificity of each RT-PCR reaction was checked by its dissociation curve. To attain our qPCR data, the samples were run on a StepOnePlus™ Real-Time PCR System using Real-time PCR mixes prepared with SYBR Green Mix solution and custom-made primers. The following program was used: 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute, for 40 cycles run at the standard 2-hour time. RNA equivalents were normalized to determine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample simultaneously. Results were analyzed by obtaining Ct quantitation values to compare the results using the ddCt method. Our control used in comparing each primer was GAPDH. We used Microsoft Excel to calculate the change in Ct values between the GAPDH Ct values and each differing primer Ct Value to obtain a percent for each sample. The standard deviation and standard error were also calculated for reference to accuracy. The percentage values obtained from the Ct values were graphed to compare levels.

**Data analysis:** All pressures were measured in reference to the atmospheric pressure as the zero 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 electrical stimulation period. Delta pressure was defined as the difference between maximum and rest pressure. The force of contraction of the EAS muscle was determined by calculating muscle stress (Tm) using the following equation: Tm = P × rm/tm, where P is the intraluminal pressure, rm is the inner radius of the EAS muscle, and tm is the EAS muscle thickness. The rm and tm were measured from the ultrasound images (measured separately using a 2 mm diameter US catheter) of the rabbit anal canal and further confirmed by
measuring these parameters in a freshly harvested specimen. EAS muscle thickness was calculated for each probe size assuming conservation of mass. The EAS muscle stress represents the average circumferential force per unit area of the circular muscle and is denoted in milli-newton per centimeter squared (mN/cm²)(30).

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

Age related changes to the EAS muscle thickness were determined using endoanal ultrasound probe and the results are shown in Figure 1. A significant (p <0.05) decrease in muscle thickness (mm) in the old rabbits (0.36; black bar) compared to the young (0.66; open bar) was observed.

Age related changes in the anal canal pressure and EAS muscle tension are shown in Figure 2A-B. With increase in probe size, there was significant increase in pressure as well as tension in both young and old rabbits. However, the pressure as well as the muscle tension was significantly (p <0.05) lower in old animals for all the probes except 3 mm. In young rabbits, the mean anal canal pressures (mmHg) recorded for 3, 4.5, 6 and 9 mm probes were 137±16, 173±15, 197±20 and 214±26 respectively. In old rabbits, the mean anal canal pressures (mmHg) recorded were 99±16, 91±9, 118±13 and 120±10 respectively (Fig 2A). Similarly, old rabbits developed lower muscle-tension (mN/cm²) for 3, 4.5, 6 and 9 mm probes (509±80, 548±57, 855±92 and 1295±106 as compared to the young animals 711±81, 1043±90, 1246±142 and 2304±274, respectively; Fig 2B).

Age related changes in the EAS muscle and connective tissue contents as determined by trichrome staining are shown in Figure 3A-B. In trichrome stained EAS sections, red color represents muscle and blue the connective tissue. In young animals, the anal canal cross section revealed healthy EAS muscle fibers arranged in a uniform pattern with relatively small amounts of connective tissue in comparison to the old animals who had scanty muscle fibers and increased connective tissue. Our image analysis of the entire cross sections showed a significant (p <0.05) reduction in the muscle content (52%) in old rabbits when compared to the young.
(72%) animals. Correspondingly, there is a significant increase in the connective tissue (52%) in
the old EAS muscle.

Next using IHC, we assessed the protein expression of important fibrosis markers (Collagen-I;
TGF-β and β-Catenin) that are shown in Figure 4. Anal canal cross sections of both young and
old animals revealed immunolocalization for all the three markers of fibrosis. However, tissue
sections from old animals revealed more intense staining for these markers when compared to
the younger animals (Figure 4).

Finally, we quantified the age-related changes in fibrogenic proteins by Western blot (Figure
5A-B) and the gene expression of these markers by qPCR studies (Figure 5C). Figure 5A
shows representative images depicting age related changes in protein levels of the three
fibrogenic proteins, β- catenin, collagen-I and TGF- β. Image analysis revealed a significant
(p<0.05) increase in all three fibrogenic proteins (Figure 5B). Our qPCR data showed a
significant (1.5 – 4 fold; p<0.05) increase in the expression of fibrosis markers in the old animals
group compared to young rabbits (Figure 5 C). Maximum (4 fold) increase was seen in the β-
catenin gene expression in the old animals (Figure 5C). A similar trend in the immune-
localization, protein expression and mRNA levels of atrophy markers (atrogin-1, MURF-1) was
observed in the aging EAS (Figure 6A-D).
DISCUSSION:

The findings of our study confirm previous reports that with advanced age there is loss of EAS muscle thickness and impairment of its function, i.e., decrease in the anal canal pressure and alterations in the EAS length-tension property. Histological data show reduction in muscle mass and excessive collagen deposition in the old rabbits. In addition, our studies show that with aging there is upregulation of major fibrogenic signaling pathways, i.e. Wnt-β catenin and TGF-β in the EAS muscle. Physiological data from our animal studies are in agreement with the previous human studies(3, 17, 37). Whitehead et al (37) in a large epidemiological study found a linear progression in the prevalence of FI with age (15% in older age group as compared to <3% in younger population) irrespective of the sex. The exact pathophysiology of increased prevalence of FI in the geriatric population is still debated. It is likely to be multifactorial in etiology in which rectal compliance, rectal mucosal inflammation, excessive amount of liquid stool and impaired anal closure mechanism play important roles. We believe though that the impaired function of muscles of anal continence is likely to be an important player. This view is supported by several epidemiological studies. Bannister et al(3) demonstrated a decrease in anal canal pressure with age. Fox et al(17) reported a reduction in the anal canal rest and squeeze pressures in the geriatric population. Our own study shows that the length-tension function of the EAS and PRM is markedly impaired in FI patients compared to age matched controls(24). Though the molecular mechanism of sphincter muscle dysfunction is still unclear, based on our findings we propose that the age-related atrophy and fibrosis of EAS muscles likely play an important role in the pathophysiology of FI.

We evaluated EAS thickness using US measurements and muscle function by determining its length-tension property, fibrosis and collagen deposit in the muscle and changes
in molecular markers of fibrogenesis. Decreased EAS muscle thickness with aging suggests muscle atrophy leading to impaired EAS muscle closure function. The 36-month-old female’s rabbits used in this study are equivalent to 65-year-old women, based on the rabbit life span. Atrophy and fibrosis of anal sphincter muscles are implicated as common pathophysiology in the age as well as injury related FI (6, 18). Recent reports indicate aberrant activation of Wnt signaling pathways in the injury as well as age-related skeletal muscle fibrosis suggesting common molecular pathways between age and injury related muscle dysfunction(9). There are several important signaling pathways that regulate skeletal muscle atrophy and fibrosis(7, 11, 22, 27, 39-41). Among these, canonical Wnt-β catenin signaling and TGF-β pathways are thought to be the major players in fibrosis. Atrogin-1 and MURF-1 are also recognized as markers of skeletal muscle atrophy(7) and our findings suggest a role for these proteins in the EAS muscle atrophy. In addition, atrogin-1 mediated muscle atrophy may involve TGF-β pathways(28).

Canonical Wnt signaling cascade involves soluble Wnt ligands interaction with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP). The later stimulates phosphorylation of disheveled (a cytosolic phosphoprotein) protein that inactivates glycogen synthase kinase 3 β (GS3Kß) phosphorylation of β catenin. With the assistance of axin, the de-phosphorylated and stable β- catenin does not undergo ubiquitination and degradation; instead it is translocated to the nucleus where it binds to the Wnt pathway effector transcription factors, T cell factor 1 (TCF-1), and lymphoid enhancer-binding factor (LEF-1), (13, 14, 33).

Wnt/β-catenin signaling is activated because of injury as well as with normal aging and is reported to play a key role in several types of injuries induced fibrosis in several organ systems, i.e., ischemia induced myocardial fibrosis, idiopathic and bleomycin induced pulmonary fibrosis, fibrosis seen in chronic kidney failure, liver fibrosis and abnormal skin wound healing(10, 16).
In all the above examples Wnts and positive regulators of β-catenin signaling are up-regulated and inhibitors of Wnt/β-catenin signaling are down-regulated. Antagonists of Wnt have been found to reduce fibrosis in the above organ systems. Akhmetshina et al (2012)(1) found that canonical Wnt signaling is critical for TGF-β-mediated fibrosis and implicated a key role for the interaction of both pathways in the pathogenesis of fibrotic diseases. Findings from our study that age related EAS muscle fibrosis is associated with upregulation of both Wnt and TGF-β-mediated signaling pathways support these interactions between major fibrosis pathways. Targeted anti-fibrotic therapy has been shown to reduce fibrosis and improve organ function(36, 42).

Our present study has some limitations. All of the old rabbits used in the present study were retired breeders. Due to logistic issues, we were unable to obtain nulliparous old female rabbits from commercial vendors. In view of the above, we can not be certain about the possible impact of child birth in these old rabbits on the observed changes. Another limitation pertains to the observation of increase in profibrotic markers. We realize that this increase does not prove the cause and effect relationship. A future study using specific antagonists such as secreted frizzled–related protein 2 (sFRP2)(32) can prove the cause and effect relationship. Withstanding above limitations, our study is the first one to demonstrate age-related changes in the sphincter muscle morphology and function using multiple parameters. In addition, we also suggest the possible role of novel fibrogenic and atrophy markers in the aging EAS muscle. If proven to be correct, our findings can have significant clinical implications, i.e., use of specific antagonists (4, 15, 21, 32, 34) to prevent age-related muscle atrophy and fibrosis in the prevention of EAS and other pelvic floor muscle dysfunction. A number of Wnt antagonists are available for systemic, oral and topical use(4), and may be useful in the prevention of age related anal
sphincter muscle dysfunction and fecal incontinence. In summary, our results suggest 

noteworthy details about aging and various muscle parameters, i.e., muscle thickness, anal canal 

pressure/tension, muscle to connective tissue ratio, and expression of fibrotic markers all of 

which imply anal sphincter weakness with aging.
**FIGURE LEGENDS**

**Figure 1. Endoanal US Assessment of Muscle Thickness:** Panel A shows representative ultrasound images of anal canal cross sections from a young and old female rabbit. Panel B–Bar graph shows mean (mm) EAS muscle thickness in young and old animals. A significant (P <0.05) decrease in the EAS muscle thickness was observed in old animals when compared to young rabbits.

**Figure 2. Anal Canal Pressure and EAS muscle tension:** (A) Anal canal pressures were measured (mm Hg) with different probe sizes (3, 4.5, 6 and 9 mm) in young and old rabbits at maximum stimulation current (6 mA). (B) EAS muscle length-tension relationship in young and old rabbits. A significant decrease (*P <0.05) in EAS muscle tension in old animals when compared to the young rabbits was observed for all probes.

**Figure 3.** (A) Masson- trichrome stained rabbit anal canal sections from young (left) and old animals (right). Histological evidence of EAS muscle atrophy and widespread fibrosis is revealed by Masson- trichrome stain in old animals. The EAS muscle is stained as red and connective tissue as blue in these images. A significant increase in the connective tissue and decrease in the muscle was observed in old animals (P<0.05) compared to the young rabbits (n=3-4 from each group).

**Figure 4: Representative photomicrographs showing immunolocalization of fibrosis markers, Collagen-I (top), TGF-β (middle) and β-catenin (lower) at different magnifications: 1X, 4X, and 10X in the EAS muscle tissue in young versus old rabbits.**

**Figure 5. Age-related changes in fibrosis Markers (A) Protein and (B) mRNA Expression:** Rabbit EAS tissue protein levels estimated by Western blot (A) followed by (B) densitometry and (C) mRNA levels of β-catenin, collagen-I and TGF-β from young and old animals.
Significant increases in the protein and mRNA levels of all three markers were observed in old animals (n=3 each). *P <0.05

Figure 6. Age-related changes to atrophy marker (A-C) protein and (D) mRNA expression
(A) Representative photomicrographs showing immunolocalization of atrophy markers (atrogin-1, MURF-1); (B) atrophy marker protein levels estimated by Western blot followed by (C) densitometry and (D) mRNA levels of atrogin-1, MURF-1 from young and old animals (n=3 each). *P <0.05

REFERENCES:


A

Young Rabbit

Old Rabbit

EAS

US

Catheter

B

Muscle Thickness (mm)

0.0
0.2
0.4
0.6
0.8
1.0

n=3

Young

n=3

Old

p < .05
A

4x

Young

Old

Trichrome

M = mucosa
S = serosa
E = EAS

Muscle
Connective tissue

B

100

% Young Old

* p < .05

72 52
28 48

Muscle Connective Tissue

Percent (%)

0
External Anal Sphincter

Collagen-1
- Young
- Old

TGF-β
- Young
- Old

β-Catenin
- Young
- Old

E = EAS, M = mucosa, S = serosa
Atrogin

Murf-1

<table>
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Atrogin
Murf-1
GAPDH

Young | Old
---|---

* p < .05

Protein (AU x1000)

mRNA Level (%)