Analysis of Fiber Type Transformation and Morphologic Changes of Small Bowel in Chronic Electrically Stimulated Canine Rectus Abdominis Muscle Stomal Sphincters

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Introduction

Present day research examining the transformation of skeletal muscle through electrical stimulation uses similar concepts to those observed by Salmons et al., who in 1981 used electrical stimulation to transform the metabolic characteristics of skeletal muscle, enabling it to perform more work over longer periods through aerobic metabolism. They showed that skeletal muscle fibers could be transformed from fast twitch (type II) fatigue-prone fibers to slow twitch (type I) fatigue-resistant fibers through training with low-frequency electrical stimulation. Applications involving the training of the graciloplasty and gluteoplasty muscular flaps with low frequency stimulation have improved upon their original design by prolonging the mechanical circumferential force generated by these skeletal muscle flaps making them more clinically useful.

The majority of research dedicated to the functional, metabolic and histologic changes which take place in skeletal muscle has been observed in in-vivo skeletal muscle experiments in small animals. In these models, the skeletal muscle remains at its optimal resting length allowing the actin and myosin filaments to fully interact, generating the most effective amount of force per square area, and thus maximizing the work capacity of the muscle. Additionally, the vascular supply to these skeletal muscle flaps is usually undisturbed with preservation of the muscles main vascular pedicle and its perforators. Understandably, the ability to maintain the optimal resting length of the skeletal muscle may influence the fiber type transformation process. Experimental cardiomyoplasty animal models and human cardiomyoplasty cadaver studies have shown that the complications associated with this procedure may be attributed to the inability to maintain the resting length of the latissimus dorsi muscle after it is wrapped around the heart, along with secondary ischemic muscular changes and the loss of flap innervation.

Other animal models using the graciloplasty have experienced similar problems with respect to disruption of the muscles resting length and distal muscular flap necrosis.

One of the potential problems encountered with a functional dynamic skeletal muscle stomal sphincter is the formation of ischemic strictures within the bowel encircled by the muscle flap. Chung et al., have shown in a chronic dog model that ischemia of the bowel around colostomies leads to ischemic bowel strictures. Other investigators have shown that acute ischemic influences on the bowel result in morphologic changes in the bowel layers including loss of the mucosa and a decrement in the muscular layers.

In-situ dynamic flap models such as our island-flap stomal sphincter offer insight in the chronic adaptive changes of the muscle fiber type transformation process caused by chronic electrical stimulation and into...
potential problems, which may arise from the dynamic physiologic changes, which take place in the small bowel, encircled by the sphincter. Therefore the purpose of this study was to: 1) determine if fiber type transformation from fatigue-prone (type II) muscle fibers to fatigue-resistant (type I) muscle fibers could be demonstrated in our chronic canine stomal sphincter model where the rectus abdominis muscle (RAM) was used to create a functional stomal sphincter, 2) assess if there was any relationship between the degree of muscle fiber type transformation and the continence times among the two different training protocols as used in Chapter 7, Part I. Intramuscular stimulation, 3) examine the long-term effects of the training regimens on the skeletal muscle fibers through histologic and volume-metric analysis over a flap stimulation period of 12 weeks, and 4) to examine the morphologic characteristics which could take place in the layers of the small bowel wall encircled by our dynamic sphincter.

Materials & Methods

In the eight male mongrel dogs (23-25 kg), as used in Chapter 7, Part I. Intramuscular stimulation, a RAM island-flap stomal sphincter was created, as previously described, by wrapping the left RAM flap around a blind loop of distal ileum (TV-loop), which was no longer in continuity with the rest of the small bowel. Both ends of the blind loop of distal ileum were matured as ileostomies on either side of the animal's abdominal wall. The island-flap sphincter remained on the left side of the abdomen and was stimulated with two intramuscular electrodes placed around the flap's intercostal nerve entry point. The sphincters were trained over 12 weeks using two different training protocols as previously described in Chapter 7, Part I. Intramuscular stimulation (Table 1, 2). Muscle biopsies were obtained pre- and post-training from the RAM sphincter. Fiber types within the RAM were assessed with monoclonal antibodies directed against the fast isoforms of myosin. Fiber volume-metric data were obtained after immunohistochemical analysis of the stained fibers with computer scanning software. Fiber histology was examined after staining with Eosin & Hematoxylin and Mason Trichrome stains. Every two weeks sphincter function (continence time) was assessed by its ability to stop the flow of saline (duration of time (minutes)) at an intraluminal TV-loop pressure of 60 mm Hg. At the completion of the study, the right control stoma, the left RAM sphincter-stoma complex and a mid portion of the small bowel wall within the TV-loop were harvested for histologic assessment.
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Quantification of Fiber Types and Histology of Skeletal Muscle
Preoperative biopsies were obtained from the untrained RAM for histology and histochemical analysis. The study was terminated at 14 weeks, which was felt to be an adequate time frame to permit fiber type transformation to take place. Post-mortem biopsy samples were collected from the skeletal muscle sphincter 1 cm from the electrode entry point. The untrained and trained RAM biopsy samples for histochemical analysis were frozen in liquid nitrogen and stored at -70°C. At the time of processing, samples were cut in 10 µm sections in a cryostat and incubated with a monoclonal antibody MY-32 (Sigma Chemical Co., St Louis, MO) against the mouse major histocompatibility complex type II. Specimen cross sections were photographed under an Olympus CKZ inverted microscope (Jacob Instrument Corp., Shawnee Mission, KS) at high magnification (100x). A clear plastic grid (5 x 15) cm was placed over the microphotograph prints and the fiber types were counted by two independent examiners. Fibers which fell on the outer grid marks were included as part of the specimen. At least 450 fibers were counted per specimen.

Specimens for histological analysis were fixed in 4% phosphate buffered paraformaldehyde (pH 7.4), paraffin embedded cross sections of 6 µm thickness were cut on a microtome in a cryostat. Specimens were then placed on cover slips and stained with Hematoxylin & Eosin and Mason Trichrome stains.

Volume-metric measurements were obtained after scanning the histochemical fiber prints stained with the monoclonal antibody MY-32 against the fast isoform of myosin into a computer. Sigma Scan Pro Software® Version 5.0 (SPSS Science Co., Chicago, IL) was used to calculate the area within individual muscle fibers.

Histology/Morphometric Measurements of Small Bowel
The island-flap stomal sphincter complex, mid-portion of the small bowel within the Thiry-Vella loop and contralateral (control) stoma were obtained after the training protocol was completed at the time of sacrifice. The tissue specimens were fixed in formalin and paraffin embedded cross sections of 6 µm thickness were cut on a microtome in a cryostat then placed on a glass slide, cover-slipped and stained with Hematoxylin & Eosin and Trichrome stains. Histologic specimens were examined under the supervision of a Board certified pathologist (DA) to determine if architectural changes were present in the specimens. An ocular micrometer was used to quantitate the thickness of the intestinal wall components: mucosa, muscularis mucosa, submucosa, circular and longitudinal muscularis propria.
Statistical Analysis
Study design was implemented with a pair of groups. A paired t test was used to compare the fiber-type transformation, fiber volume and bowel layer thickness morphology between untrained and trained skeletal muscle. Data are represented as mean ± SEM. Significance was attributed to P values < 0.05.

Results
The percentage of type I and II muscle fiber types was determined immunohistochemically (Fig. 1). After continuous training over 12 weeks using training protocol A, the percentage of type I muscle fibers increased from a mean of 30.8 % ± 1.8 to 73.8 % ± 10.4, while the type II fibers decreased from 69.3 % ± 1.8 to 26.3 % ± 10.4. Training protocol B resulted in an increase of type I muscle fiber transformation from a mean of 39.3 % ± 2.3 to 77.3 % ± 6.8 with a concomitant decrement of type II muscle fibers from 60.8 % ± 2.3 to 22.8 % ± 6.8 (Table 1). Fiber diameters and volume were reduced after the skeletal muscle was trained. Continuous stimulation using training protocol A and B resulted in a decrement of 32% of the total

<table>
<thead>
<tr>
<th>Protocol A</th>
<th>Protocol B</th>
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<tr>
<td>Untrained</td>
<td>Untrained</td>
</tr>
<tr>
<td>RAM (n=4)</td>
<td>RAM (n=4)</td>
</tr>
<tr>
<td>% Type I Fibers</td>
<td>% Type I Fibers</td>
</tr>
<tr>
<td>30.8 ± 1.8*</td>
<td>39.3 ± 2.3*</td>
</tr>
<tr>
<td>% Type II Fibers</td>
<td>% Type II Fibers</td>
</tr>
<tr>
<td>69.3 ± 1.8*</td>
<td>60.8 ± 2.3*</td>
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* p < 0.05 (untrained vs. trained); Data are expressed as percentage of specimen fiber types ± SEM.

<table>
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<tr>
<th>Fiber Volume</th>
<th>Untrained RAM</th>
<th>Trained RAM</th>
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<tr>
<td>(Pixel units)</td>
<td>758.6 ± 17.6*</td>
<td>519.1 ± 20.6*</td>
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* p < 0.01 (untrained vs. trained); Data are presented as mean ± SEM.
fiber area as shown in Table 2. There was a statistically significant difference between the fiber volumes in pixel units between the untrained and trained muscle fibers (P < .001).

The fiber morphology between the untrained and trained skeletal muscle is depicted in Figure 2. In the untrained skeletal muscle specimens, the fibers are polygonal in shape with very little connective tissue or fat in between the fibers. After training, the fiber size (diameter) decreased, the general appearance of the fibers varied little without structural changes and there was

**Figure 1**

Immunohistochemistry of untrained and trained skeletal muscle fibers stained with an antibody against the fast forms of myosin. **A)** Untrained RAM fibers, fibers are predominately of the fast fiber type. **B)** Trained RAM fibers obtained from the island-flap sphincter after 3 months of training, the majority of fibers are of the slow fiber type.
Figure 2
Histology of skeletal muscle fibers (magnification 100x, Bar 0.05 mm). A) H&E stain of untrained rectus abdominis skeletal muscle, note large fiber diameter with peripheral cell body nuclei. B) H&E stain of rectus abdominis skeletal muscle sphincter biopsy after 3 months of training, fiber diameter is smaller than untrained muscle, nuclei appear crowded due to smaller fiber diameter. C) Trichrome stain of trained rectus abdominis muscle sphincter wrap around small bowel, note minimal fibro-fatty deposition between atrophied skeletal muscle fibers.
Table 3  Morphometric Measurements of Bowel Layers

<table>
<thead>
<tr>
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<th>Stoma</th>
<th>Stomal Sphincter</th>
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<tbody>
<tr>
<td>Mucosa</td>
<td>1.04 ± .09</td>
<td>1.16 ± .14</td>
</tr>
<tr>
<td>Muscularis Mucosa</td>
<td>.08 ± .01</td>
<td>.08 ± .01</td>
</tr>
<tr>
<td>Submucosa</td>
<td>.43 ± .08</td>
<td>.30 ± .06</td>
</tr>
<tr>
<td>Circular Muscle</td>
<td>.63 ± .09</td>
<td>.57 ± .07</td>
</tr>
<tr>
<td>Longitudinal Muscle</td>
<td>.25 ± .03</td>
<td>.25 ± .04</td>
</tr>
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</table>

* Data are expressed as mean thickness of bowel layers (mm) ± SEM, n = 7.

Figure 3
Small bowel canine biopsies, (magnification x100, Bar 0.10 mm).
A) H&E stain of small bowel biopsy from mid-portion of Thiry-Vella loop with undisturbed bowel layers. B) H&E stain of small bowel biopsy encircled by dynamic sphincter with healthy mucosa and unimpressive changes in the remaining bowel layers. C) Trichrome stain of small bowel encircled by dynamic sphincter without evidence of gross fibrosis of the small bowel layers. D) H&E stain of contra-lateral stoma small bowel layers.
a minor increase in the endomysial tissue between the fibers. Microscopically there was no evidence of damage to the fibers or degenerative changes.

The continence time for the skeletal muscle sphincters measured in minutes (mean ± SEM) for training protocol A averaged 1.3 ± 0.5 minutes at 10 weeks in contrast to the revised training protocol B which averaged 229 ± 10 minutes over the same time period, (P < 0.05). In Figure 4, Chapter 7, a comparison of RAM sphincter continence time (hours) between Group A and Group B at different time-points (weeks) after start of electrical stimulation are depicted. Data are presented as mean ± SEM. (* p< 0.05 vs. Group A)  

Morphometric analysis of the individual bowel layers within the Thiry-Vella loop and the sphincter were similar in thickness (p > 0.05) when measured with a micrometer (Table 3). Histologically, the layers of the bowel which were encircled by our dynamic stomal sphincter including the mucosa, muscularis mucosa, submucosa, and muscularis propria were without architectural changes including neither ulcerations nor necrosis. Trichrome stains did not reveal any evidence of fibrosis within the bowel layers (Fig. 3).

Discussion

The dynamic RAM island-flap sphincter designed in this experiment for stomal continence relies upon the fatigue-resistant properties associated with type I skeletal muscle fibers. These fibers have been shown in previous experiments to contain a greater percentage of mitochondria, rely more heavily on oxidative metabolism, contain a higher capillary density, and have reduced glycolytic activity. The above skeletal muscle properties are advantageous with respect to promoting fatigue-resistance when increased demands are placed on skeletal muscle. Our previous experimental work with our dynamic island-flap stomal sphincter created from the RAM showed that the RAM in the canine consists predominately of fatigue-prone (type II) fibers (approximately 65%) with the remaining fibers of the fatigue-resistant type (type I) in origin. After studying many other skeletal muscle dynamic flap designs we felt that the maximal work potential of our flap could be achieved by: 1. Maintaining the innervation of the flap enabling it to be adequately trained. Previous experiments using a free gracilis muscle flap to achieve stomal continence in a canine model were largely unsuccessful due to flap denervation atrophy which lead to poor flap performance. This concept is also appreciated by other investigators who have attributed the variable results of the cardiomyoplasty to loss of the flap’s innervation distally which impaired the work performance of such flaps. The maintenance of adequate flap perfusion by preservation of the arterial supply and venous drainage to all regions of the flap. Prior research on cardiomyoplasty and graciloplasty have shown that poor perfusion
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to the distal part of the flap has a detrimental effect on flap performance and fiber phenotypic changes.\textsuperscript{23,24} 3. Achieving adequate stretch of the flap’s muscular fibers to attain physiologic overlap of the actin and myosin filaments thus optimizing the skeletal muscle’s contractile characteristics. Williams and associates have shown that muscle stretched to its optimal resting length along with physiologic stimulation results in the least amount of connective tissue deposition within the skeletal muscle and leads to improved muscular work capacity and performance.\textsuperscript{25,26}

More recently, investigators have attempted to preserve the relative portion of type IIA intermediate muscle fibers (a subset of type II fibers) which have superior forceful contractile qualities when compared to type I fibers along with acceptable fatigue-resistance properties.\textsuperscript{9,27} Although there was no appreciable difference between the amount of type I fibers transformed using the two different training regimens, the continence times were dramatically different between the two groups when the sphincter was stimulated to contract against an intraluminal bowel pressure of 60 mm Hg. Training protocol A with a mean continence time of $1.3 \pm 0.5$ minutes was inferior to protocol B’s mean continence time of $229 \pm 10$ minutes after 10 weeks of training ($P < 0.05$). One possible explanation for the difference between the two protocol’s continence times is that the relative proportion of type IIB and IIA fibers might have been different between the two training protocols with a higher percentage of type IIA fibers in the animals trained with protocol B. Our monoclonal antibody stain was unable to differentiate between these two types of fibers. Type IIA fibers which exhibit superior fatigue-resistance and adequate contractile properties when compared to type IIB fibers have been shown to be a stable inducible phenotype. The conversion and maintenance of type IIA fibers is highly dependent on the training protocol used.\textsuperscript{9,27} Lower frequency stimulation was found to play a significant role in fiber type conversion and maintenance of type IIA fibers in a study by Sutherland et al.\textsuperscript{27} In our experimental training protocol B we used an initial lower frequency of 14 Hz during the early stimulation period over 8 weeks when compared to training protocol A with its 25 Hz frequency. This may have influenced the relative proportion of type IIA fibers between the two groups. An additional explanation may be attributed to the relative amount of change and stability of the enzymatic cellular processes present in the muscular fibers oxidative metabolism. Kwong and associates have shown that the inducible enzymes necessary for aerobic metabolism are variable and may proceed the fiber transformation process in skeletal muscle fibers.\textsuperscript{28} The ability of skeletal muscle to maintain a contraction in an efficient manner after transformation of the skeletal muscle’s fiber type is complete, may depend on the relative proportion of key enzymes in the citric acid cycle, fatty acid oxidation pathway and respiratory chain.\textsuperscript{11} Perhaps in our study, both stimulation
protocols were able to transform the phenotypic characteristics of the muscle through changes in the myosin filaments, while protocol B was superior at producing and maintaining a greater proportion of those enzymes needed to generate a more efficient contraction. The volume-metric data suggests that training resulted in a decrement of muscle fiber size by 32%. The reduction in fiber size may be beneficial through the reduction in the diffusion distance between the capillaries and the cells mitochondria, thus potentially increasing the oxidative metabolic capacity of the muscle. The training protocols used in this experiment were adequate at transforming the fibers from type II to type I fibers, but perhaps unable to maintain the relative percentage of the intermediate type IIA fibers which are generally larger in diameter when compared to type I fibers. Duan et al., have shown that fiber diameter may be influenced by the duration of stimulation and rest used in the training protocols. Intermittent stimulation with more frequent rest may result in fibers more characteristic of type IIA fibers which tend to retain their size and more closely resemble the fast-twitch muscle fibers in diameter, while preserving the oxidative fatigue-resistant properties of type I skeletal muscle.

Histologic analysis of the sphincters also correlates with the volume-metric data results both of which showed a decrease in the fiber diameter resulting in less distance between the fiber’s nuclei. The Mason Trichrome stain did not show significant accumulation of fibrotic tissue between the skeletal muscle fibers. Atrophy was apparent without obvious damage to the skeletal muscle fibers. The lack of significant fibrosis between the skeletal muscle fibers may be attributed to the degree of stretch found within the island-flap’s wrap and the generous blood supply provided by the deep inferior epigastric artery. Oakley et al., demonstrated in a latissimus dorsi muscle goat model that loss of the flap’s resting tension alone seemed to contribute substantially to the degree of muscular flap damage and connective tissue infiltration. When the flap was subjected to a loss of its resting length along with partial devascularization and constant training, the added effect of all three parameters resulted in a higher degree of muscular flap damage with fat and connective tissue infiltration. Similar architectural characteristics have been observed in the latissimus dorsi muscles of cardiomyoplasty patients on post mortem exam with replacement of muscle fibers by fibrofatty tissue. Our model allows the skeletal muscle fibers to remain stretched around the bowel circumferentially. This is unlike the graciloplasty in which the muscle is wrapped around the bowel in a twisted configuration, which may influence the contractile characteristic of the wrap and the degree of fiber stretch and ultimately the deposition of collagen between the fibers.
With prolonged external compression of an intestinal stoma it is reasonable to assume that the sphincter has the potential to cause ischemic trauma to the small bowel that it encircles. Several investigators have noted the influence of an ischemic insult on bowel. Cohen et al., has shown in a canine model early changes in the bowel layers including hemorrhage into the mucosa, congestion of the mucosa and submucosa with subsequent mucosal sloughing with balloon occlusion of the superior mesenteric artery over 3 hours. Chung and colleagues have investigated and determined in a canine model that bowel ischemia is proportional to the length of bowel devascularized and this ischemic insult influences the anastomotic diameter of colostomies. In their model the ischemic event lead to changes in the architecture of the bowel including fibrosis of the muscularis and submucosal layers. In our stomal sphincter model the terminal ileal vascular arcade is ligated to accommodate the skeletal muscle wrap thus the bowel within our sphincter must be supplied by intramural collateral flow. Armstrong and associates have shown in a canine model that ligating the vascular arcade over 4 cm of bowel (as in our model) results in a decrement of blood flow from 32.4 ml/100 gm/min to 15.6 ml/100 gm/min or intramural blood flow falls with the square root of the distance from the inflow source. We suspect that flow to the bowel in our model may be lessened initially. This influenced our decision to begin the stimulation protocol 2 weeks after the creation of our sphincter. However, with chronic flap stimulation we felt that our sphincter could potentially have an adverse effect on the bowel from prolonged compressive flap ischemia.

Morphometric and histologic data of the bowel layers did not identify any gross differences between the control bowel and bowel encircled in the stomal sphincters. The thickness of the bowel layers was similar between the two groups. The mucosal layer, which is the most susceptible to ischemic changes, remained healthy in the sphincter group. Bonakdarpour and associates have shown in a semi-chronic canine model that when the bowel was subject to an ischemic insult afflicting a 65-105 cm segment of bowel over a 2-3 week period varying degrees of mucosal ulceration and necrosis occurs. In our sphincter model the convoluted bowel architecture of the mucosa was preserved with the normal compliment of crypts and secretory (goblet) cells. There was no evidence of mucosal necrosis or ulceration. The muscularis mucosa was unchanged between the control and sphincter groups and there was no evidence of muscular atrophy within this layer. The submucosal layer which is naturally composed of connective tissue and has been shown in numerous studies to become fibrotic with chronic ischemic insults was also histologically unchanged between the two animal groups in our study. The largest smooth muscle layer of the bowel, the muscularis propria includes the circular and longitudinal muscles. Previous studies in dogs have shown that the cross sectional surface area and thickness of the circular and longitudinal
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muscles of the small bowel is parabolic with the greatest thickness of these layers occurring in the proximal and distal segments of the bowel. We also were unable to detect any significant differences in the thickness within these layers between the control and sphincter groups. These layers may be more resistant to ischemic insults because of the relative thickness of these tissue layers and their close proximity to the mesenteric collateral blood flow. Also, the parallel orientation of the island-flap’s muscular sphincter probably exerts a more even distribution of pressure over the bowel lessening the risk of ischemic damage to the bowel wall. This is unlike the gamma wrap of the conventional graciloplasty procedure for fecal incontinence which has been found to produce colonic fistulas from the force exerted by the gracilis tendon along the colonic wall and bowel ischemia.

Conclusion

In conclusion, it is possible to achieve fiber type transformation from fatigue-prone (type II) muscle fibers to fatigue-resistant (type I) muscle fibers in this unique canine island-flap model using the RAM to construct a stomal sphincter. To achieve a successful functional sphincter it is critical to maintain the vascular perfusion and neuronal innervation of the flap, the loss of which may contribute to muscular atrophy and poor flap performance. The training protocol used to convert the skeletal muscle sphincter to more favorable fatigue-resistant properties may profoundly influence the contractile characteristics of the flap while minimally affecting the degree of fiber type transformation. Fiber atrophy, which was seen in this study, is common when skeletal muscle is transformed from type II to type I fibers. Histologically minimal fibro-fatty deposition between the muscle fibers was seen. In addition, our dynamic island-flap sphincter constructed from the RAM and trained over a 3-month period does not result in ischemic damage to the bowel wall that it encircles. There were no significant architectural differences in the layers of the bowel wall encircled by the stomal sphincter, which suggests that the collateral intramural perfusion was sufficient to maintain the integrity of all the bowel layers.
References

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