DEFINING THE HISTOPATHOOGICAL CHANGES INDUCED BY NON-ABLATIVE RADIOFREQUENCY (RF) TREATMENT OF FAecal INCONTINENCE – A BLINDED ASSESSMENT IN AN ANIMAL MODEL

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Maciej Murawski: substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published.

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ABSTRACT

Aim: Non-ablative radiofrequency (RF) sphincter remodeling has been used to treat gastroesophageal reflux disease (GERD) and faecal incontinence (FI). Its mechanism of action is unclear. We aimed to investigate the histomorphological and pathophysiological changes to internal (IAS) and external anal sphincter (EAS) following RF.

Method: An experimental FI model was created in 12 female pigs: eight underwent RF 6 weeks following induction of FI (FI+RF) and four were untreated (UFI). Four animals served as controls (CG). Two blinded pathologists examined all H&E and trichrome stained slides.
Results: Compared with the UFI group, histological examination of the internal anal sphincter (IAS) in the FI+RF group demonstrated an increased smooth muscle (SM)/connective tissue ratio (77.2% vs. 68.1% p <0.05) and increased collagen-I compared with collagen-III content (67.2% vs. 54.9% [p<0.001]). The RF+FI group exhibited greater SM bundle thickness compared with the UFI group (SM width: 486.93 vs. 338.59 um; p<0.01 and height: 4384.4 vs. 3321.0 um; p<0.05). The external anal sphincter (EAS) of the FI+RF animals showed a significantly higher Type I/II fibre ratio (33.5% vs. 25.2%; p=0.023) and fibre type I diameter (67.2 vs. 59.7um; p<0.001) compared with the UFI group. Post-RF manometry showed higher basal (18.8 vs. 0 mmHg) and squeeze (76.8 vs. 12.4 mmHg; p<0.05) anal pressures. After RF treatment, the number of interstitial cells of Cajal was significantly reduced compared with the UFI and CG groups [0.9 (FI+RF) vs. 6.7 (UFI) vs. 0.7 (CG)/mm²; p<0.001].

Conclusion: Non-ablative RF in an animal model appeared to induce morphological changes in the IAS and EAS leading to an anatomical state reminiscent of normal sphincter structure.

Key words: faecal incontinence, radiofrequency anal remodeling, sphincter morphology

WHAT DOES THIS PAPER ADD TO THE LITERATURE?
The study is the first to demonstrate the morphological changes following radiofrequency in the internal and external anal sphincter in an animal model of faecal incontinence.
INTRODUCTION
Faecal incontinence (FI) is a common affliction associated with significant psychological disability and social isolation. [1-4] Non-ablative radiofrequency smooth muscle (SM) remodeling (SECCA®; Mederi Therapeutics Inc., Norwalk, CT) has been successful in treating FI and the lower oesophageal sphincter (Stretta®; Mederi Therapeutics Inc., Norwalk, CT) for the treatment of gastroesophageal reflux disease (GERD). The treatment was initially used in FI by Takahashi et al and Efron et al [5,6] and has proven to be safe and effective over 60 months after treatment. [6-10] A recent publication has, however, questioned its efficacy and durability suggesting that greater understanding of the mechanism of action is needed. [11] SECCA® has been reported to result in significant improvement in anal sphincter function and to restore anorectal sensitivity and rectoanal coordination. [10] A recent double-blind randomized crossover study of RF for gastro-oesophageal junction specifically excluded fibrosis as the primary mechanism for the improvement. [12]

The physiological mechanism responsible for faecal continence requires intact neural pathways and adequate function of the SM of the internal anal sphincter (IAS) and the skeletal muscle of the external anal sphincter (EAS). The aim of the present study was to determine the morphological changes in the anal sphincters and a cellular pathway represented by the interstitial cells of Cajal (ICC) in an animal model.

METHOD
The Agriculture University Animal Care and Use Committee approved the study protocol and experiments were conducted in accordance with the guidelines for the care and use of laboratory animals. The study cohort consisted of a group of 16 white female pigs (mean age...
181 days; weight 160 lb) randomly assigned to three different groups as follows: 1) FI+RF (FI+RF) [n=8] 2) untreated FI, (UFI) [n=4] and 3) control [non-FI+non-RF (CG) [n=4].

All procedures were performed under general anaesthesia. FI was created by a 4cm anal dilatation, left pudendal nerve (PN) destruction (1.0 ml of 50% ethanol injection to PN under nerve stimulation guidance), and right lateral sphincterotomy of both sphincters. [13]

Although the animal model of FI had not been validated in other studies, the effectiveness of nerve destruction and sphincter division was confirmed in all animals by palpation and by anorectal manometry performed before and 20 minutes after the surgery and 4 weeks later. Six weeks after the induction of FI, the standard SECCA® protocol of four needles deployed into four quadrants at five levels for a maximum of 80 RF energy application points was employed in eight of the twelve animals with FI. [6] Anal manometry was performed in ketamine-sedated animals using a solid-state probe and a portable manometry system (Sphincterometer; MSM ProMedico, Aachen, Germany) before and after the operation to produce FI and at four weeks later. As shown in Figure 1, destruction of the right pudendal nerve (PN) was performed using the modified George method for PN identification (anal contraction in response to electrical stimulation). The aim of this procedure was to create a mixed model of anal incontinence. Measurement of basal (BAP) and squeeze anal pressure (SAP) induced by perianal skin needle stimulation were recorded at each time interval. All animals were sacrificed three months after RF treatment. Manometry was repeated immediately before death.

**Histopathological study**

Immediately after death the anorectal tissues were excised and labelled with the number of the animal. Perpendicular sections of each quadrant were obtained (Figure 1) and the tissue was

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fixed in formalin. Sections from each anal quadrant were taken for evaluation, but for the purpose of this study, we evaluated only the specimens from the right posterior and lateral quadrants, which was the region innervated by the right PN which had been destroyed) and to avoid the possible influence of scar tissue due to the left sphincterotomy on the morphology of the IAS. Samples were also placed in liquid nitrogen and cryopreserved for frozen section. The following parameters were recorded and semiquantitatively assessed by image analysis (Multiscan Base Software, V.14.02 and Metamorph 7.0; Axio Vision Rel. 4.8.3):

**Internal anal sphincter**

1. **Smooth muscle (SM) height and width, SM and connective tissues percentage and the SM/connective tissue ratio**

The SM/connective tissue percentage was assessed by one of the pathologists (MB) by image analysis (Metamorph.7.7 edition). Trichrome stained sections from every block were examined and the corresponding percentage of SM and connective tissue areas was obtained under low magnification (Olympus U-SDO microscope, Japan - x1000; SM: red; connective tissue: blue). The median value for each component was then calculated and used to estimate the ratio. Muscle fibre height and width were measured in microns by image analysis (Multiscan V. 14.02) by the second pathologist (RJ) who assessed 300 smooth muscle fibres (Nikon E600 light microscope Japan; H&E - x1000) from which the median value was calculated.

2. **Connective tissue septal width and collagen composition**

The septal width of collagen I and III was semi-quantified in the formalin fixed tissue by a standard immunoperoxidase technique (collagen-I (AB6308, prediluted, Abcam), collagen-III
The reaction was visualized using the NovoLink™ Polymer Detection System (Leica, Germany) and sections were analyzed using a Zeiss Axio Imager A 2 light microscope (x1000) (Zeiss, Germany). The proportion of collagen I and collagen III was then quantified by image analysis (Multiscan V. 14.02) and median values were calculated.

3. Interstitial cells of Cajal (ICC)

The number of ICC was assessed by CD117 immunohistochemical staining of formalin fixed tissue (Cellmarque, prediluted) using standard immunohistochemical techniques. Ten high power fields (x4000) were examined and the cells were counted by image analysis (Interactive Image Processing System (IPS). The median number of ICC/mm² was then calculated for comparison amongst groups.

4  External anal sphincter

Number and ratio of types I/II skeletal muscle cells

A modified combined method of NADH-tetrazolium reductase activity and immunohistochemical determination of slow myosin heavy chain on the same section was employed to distinguish muscle fibre types [Type-I (red, slow-oxidative), Type-IIA (intermediate, fast-oxidative-glycolytic), and Type-IIB (white, fast-glycolytic)]. [14,15] EAS sections were air dried and incubated with medium for NADH-tetrazolium reductase determination. [16] Immunohistochemical staining with monoclonal antibodies against the skeletal slow myosin heavy chain was performed (clone WBMHCs Leica, Germany) on the same section. All reactions were visualized using the NovoLink™ Polymer Detection System (Leica, Germany). A minimum of 300 fibres was counted per section using a NIKON E600
light microscope via image analysis (Multi Scan V.14.02). The median values were then calculated.

**Determination of myosin heavy chain isoform**

Myosin heavy chain isoform was determined on EAS lysates by electrophoresis using a trichrome stained 8% polyacrylamide gel using a protein marker (PageRuler™ Plus Prestained Protein Ladder, Thermo Scientific, Rockford, IL)

**Statistical analysis**

Standard histology and immunohistochemistry evaluations were independently performed by two pathologists. Changes in SM bundles including height, width, number of SM cell/cross sectional area, and SM/collagen content in the IAS were compared between the UFI, FI+RF, and CG groups using two-way analysis of variance. A split-plot design compared bundles and septal thickness, myocyte CSA, and collagen content between groups by designating region and measurement time points as the main effects. Since each analysis was undertaken to compare two groups, paired comparison was deemed appropriate. The results are presented as mean ± SE, with a p-value <0.05 considered to be statistically significant.

**RESULTS**

**Internal Anal Sphincter**

1. **SM height and width, and SM/connective tissue ratio (Table 1)**

The UFI animals had significantly lower SM bundle height and width compared with the FI+RF and CG groups (3321.0 vs. 4384.4 vs. 4640.2 and 338.59 vs. 486.93 vs. 532.0 µm [p<0.05]) (Figure 2). A decreased SM/connective tissue ratio was found in the UFI group compared with the FI+RF and CG groups (2.1 vs. 3.8 vs. 4.2 [p<0.05]).
2. IAS width of connective tissue septae and collagen composition

The width of connective tissue septae was significantly greater in the UFI compared to the CG group (57.13 um vs. 44.2 um [p<0.05]). Following RF, the width of the connective tissue septae decreased to values comparable to those in the CG group (47.7 um vs. 44.2 um) (Figure 3). In the UFI group the percentage of collagen-I within the septae was significantly lower than in the FI+RF and CG groups (57.9% vs. 67.2% vs. 77.2% [p<0.05]).

3. Interstitial Cells of Cajal in the internal sphincter

Following RF treatment, the number of ICC was significantly reduced compared with UFI animals and CG ([0. 9 (RF) vs 6. 7 (UFI) and 7.2 (CG)/sqmm; p<0.001] (Figure 4).

4 External Anal Sphincter

Type I/II skeletal muscle ratio

RF treatment increased the Type I/II skeletal muscle fibre ratio compared to the UFI group (1.2 to 1.0; p=0.023). No significant difference was noted in the percentage of Type I fibres between the FI+RF and CG groups (33.5% vs. 34.4%). The diameter of the Type I fibres did not significantly differ between the FI+RF and CG groups (67.2 um vs. 68.1 um) whereas the UFI group showed smaller fibres (59.7 um; p=<0.05) (Table 2).

Determination of SM heavy chain isoform

An increase in the size and percentage of Type-I EAS muscle fibres was correlated with an increase in myosin Type-I band in SDS page analysis of heavy chain myosine in EAS lysates (Table 2).
Anal Manometry

Confirmation of successful induction of FI was demonstrated by a fall in the basal anal pressure (BAP) to 0mmHg and squeeze anal pressure (SAP) to 12.4±2.2mmHg (p<0.05).

After RF anal manometry showed higher BAP and SAP in the FI+RF group of 18.8±4.2mmHg and 76.8±12.4mmHg. These pressures were not significantly different from those in the CG (28.8±6.8 and 97.6±16.2 [p<0.05]) (Table 3).

DISCUSSION

The SECCA® procedure for FI is based on the administration of temperature-controlled RF energy to the anal canal. The mechanism of action of the SECCA® procedure has been attributed to the recovery of the sphincter function as well as anorectal sensation and coordination through C and A delta afferent fibers. Furthermore, previous studies have demonstrated increased BAP and SAP post-treatment.

Study of the histopathological events associated with RF might help the understanding of why improvement of FI has been reported by some authors. This is the first study to do so and care was taken to try and mimic in an animal model the situation faced by patients with FI. The creation of incontinence in the model was confirmed by physical examination of the animals and the decreased resting and squeeze anal pressures seen on manometry and the morphological changes of the sphincters that would be expected after denervation, although the model was inevitably limited since no subjective statement from the pigs was possible. Although we anticipated that anal stretching would have lead to fragmentation of the IAS, we were unable to confirm this hypothesis from the morphology study. This was probably because the study focused on the small portions of the IAS and not the whole muscle.
The findings suggest that RF improves IAS function by inducing hyperplasia and hypertrophy of the SM fibers, as was shown by increased SM bundle size and SM fibre numbers compared with animals which did not receive RF. These data are similar to the changes described in a mouse model of myocardial remodeling following RF in which myocyte hyperplasia and hypertrophy were observed in myocardial tissue away from the main target area of injury [14]. Similarly, in a canine model with iatrogenically induced gastroesophageal reflux, RF led to marked thickening of the muscularis propria compared with an untreated group [15]. In the current study, IAS SM bundle size, which was decreased in the UFI group, significantly increased after RF to reach values equivalent to those in the CG group.

Although the pathophysiological mechanism of this is unclear, a possible explanation is linked to structural modifications in the heat shock protein 27 (HSP27) which is present on the surface of SM cells. It has been demonstrated that heat stress induces phosphorylation of this molecule triggering polymerization of actin, which could lead to hypertrophy of the myocytes with consequent expansion of the SM layer. [16,17] HSP27 is also responsible for stimulating cell migration, which could also account for the increased numbers of SM cells. [16,17]

Interstitial cells of Cajal have been shown to play a critical role in gastrointestinal motility. This concept is supported by the association of a variety of conditions characterized by constipation and absence of and/or defects in ICC [18,19]. The IAS ICC are responsible for maintaining basal tone. This function is possibly achieved through interconnecting networks with the SM cells and the myenteric and submucosal plexuses. [20] Furthermore, it has been postulated that the ICCs mediate the inhibitory innervation of the rectoanal reflex, but, the mechanism by which this inhibitory effect takes place is poorly understood. [21] Piotrowska et al [18] demonstrated a reduced number of ICC in patients with constipation related to
achalasia of the IAS. The association of ICC abnormalities in FI has not been previously addressed and the present study is the first to describe a reduction in the number of ICC following RF. The significance of this finding requires further research, but it is possible that IAS injury leads to a proliferation of ICC with subsequent disturbance of the rectoanal inhibitory reflex that could contribute to the development of FI. Certainly, with the use of CD117 immunostaining we observed an increased number of ICC in the UFI animals compared with those having RF or no intervention. It has been suggested from work in an animal model that the increased transient lower oesophageal relaxations (TLESRs) which occur in gastroesophageal reflux disease are mediated by ICC. di Baise et al [22] reported a significant reduction in TLESR after RF. A similar phenomenon may be responsible for an improvement in anal pressure as occurred in our study, as transient internal sphincter relaxations (TIASRs) have been observed in FI patients. [23]

An underlying network of collagen fibres is critical to provide the anal sphincters with structural support for contraction and elasticity, but excessive collagen deposition would result in fibrosis with interference of normal function. In our model histological examination of the IAS retrieved from the UFI animals revealed marked fibrosis represented by thickened septae and increased connective tissue/SM fibre ratio. In contrast the FI+RF animals showed values equivalent to the controls. Although the mechanism by which RF diminishes fibrosis is not completely understood, it has been shown that the heating of collagen, as occurs during RF application, leads to a breakdown of the heat labile intramolecular crosslinks of the triple helix and subsequent protein denaturation, resulting in collagen shrinkage. [24] The ability of RF to induce collagen shrinkage is well known and several medical treatments that vary from mitral valve disorders to orthopedic conditions are based on this property. [25,26] The quality of the connective tissue is in part dependent on an adequate collagen I/III ratio. Collagen I
provides tensile strength, decreased collagen I/III ratio has been linked to reduced mechanical stability. In this regard, RF has been shown to promote Collagen I synthesis increasing the collagen I/III ratio. [24] In our study, the percentage of collagen-I within the septae was significantly lower in the UFI group than in the controls and conversely there were no significant differences between the RF and controls.

It was previously thought that SECCA® mainly affects IAS remodeling, but the present study showed that the EAS is also modified. The EAS predominantly contains Type-I slow-twitch fibres with a smaller number of Type-II fibers, reflecting the capacity of this muscle to produce sustained contractions and to react under stress with a rapid increase in tension. [27] The size and percentage of Type-I EAS fibers were significantly decreased after denervation in the UFI group compared with the controls.

Interestingly RF treatment also appeared to reverse the reduction in Type-I EAS fibers almost to normal values and to increase the percentage and size of Type-I EAS muscle fibers, as supported by the SDS page analysis of heavy chain myosine in EAS lysates. This improvement in EAS remodeling may be explained by Type-I fibre hypertrophy and hyperplasia due to either a paracrine response related to micro-damage of the IAS or possibly reduced energy transmission and micro-damage of the EAS analagous to the myocardial muscle response previously mentioned. Alternatively the effect may be due to successful reversal of a reduced number of Type-II EAS fibres after RF similar to muscle changes during reinervation [28,29]. These two potential pathways are supported by the observed increase in size and number of Type-I EAS fibres after RF. Although formal reports addressing the effect of RF on nerve structures have not been conducted, it is possible that this therapeutic modality actually stimulates nerve regrowth after injury.

In conclusion this animal model of FI has shown that non-ablative RF to the sphincter muscles produced a number of potentially beneficial morphologic and compositional changes.
changes, without evidence of scar formation. These data suggest that significant sphincter muscle restructuring rather than scarring occurs after RF treatment.

REFERENCES


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Table 1. Internal anal sphincter: comparison between smooth muscle and connective tissue components, width and height of smooth muscle layer and percentages of collagen I and III

<table>
<thead>
<tr>
<th></th>
<th>IAS</th>
<th>UFI</th>
<th>FI+RF</th>
<th>CG</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Collagen I</td>
<td>54.93+/-2.54</td>
<td>67.18+/-2.19</td>
<td>77.2 +/-2.2</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>% of Collagen III</td>
<td>36.82+/-1.63</td>
<td>25.16 +/-1.84</td>
<td>22.8 +/-3.2</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>% of muscle/bundles</td>
<td>64.95+/-0.8</td>
<td>79.72+/-0.9*</td>
<td>82.64+/-0.9</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>% of connective tissue /bundles</td>
<td>35.05+/-0.6</td>
<td>20.28+/-0.9*</td>
<td>17.36 +/-0.8</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>SM bundles height</td>
<td>3321.0+/-205.9</td>
<td>4384.4+/-261.6*</td>
<td>4640.2 +/-226.2</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>SM bundles width-µm</td>
<td>338.59+/-32.2</td>
<td>486.93+/-22.57*</td>
<td>532.0+/-24.6</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Septum width -µm</td>
<td>57.13+/-1.76</td>
<td>47.07+/-1.48*</td>
<td>44.2 +/-1.72</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

IAS=internal anal sphincter; FI=faecal incontinence; RF=radiofrequency; NS=not significant; UFI=untreated faecal incontinence; FI+RF= standard RF 6 weeks following induction of FI; CF=control group; SM=smooth muscle.

After RF muscle bundle size increased significantly compared to the FI alone group (*) and did not differ from controls. Muscle bundles were separated by large septae. In FI animals the mean width of septae was significantly greater than controls and was significantly decreased.
after RF. The changes in the size of SM bundles and septae were also reflected by the percentage of IAS muscle/connective tissue.

Table 2. External anal sphincter

<table>
<thead>
<tr>
<th>FIBRE</th>
<th>Control Group (CG)</th>
<th>UFI GROUP</th>
<th>FI+RF GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYPE</td>
<td></td>
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</table>

Percentage (%) of EAS fibres according to type

| Type I   | 34.4+/- 2.3 | 28.2+/-1.4 | 33.5+/-1.9 | <0.05 |
| Type IIA | 26.4+/-3.2  | 34.1+/-1.6 | 28.2+/-1.2 | <0.05 |

Diameter (µm) according to type

| Type I   | 68.1+/-1.2  | 59.7+/-1  | 67.2+/-0.8 | <0.05 |
| Type IIA | 63.2+/-1.0  | 60.7+/-1  | 62.2+/-1  | NS    |

Percentage (%) composition of myosine heavy chain isoforms

| Type I   | 36.2+/-2.8  | 24.2+/-1.1 | 35.1+/-1.13 | <0.05 |
| Type IIA | 24.8+/-1.1  | 37.7+/-1.3 | 25.8+/-0.9  | <0.05 |

UFI=untreated faecal incontinence; FI+RF= standard RF 6 weeks following induction of FI; CF=control group; SM=smooth muscle; EAS=external anal sphincter; FI=faecal incontinence; RF=radiofrequency; NS=not significant

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Table 3: Anal manometry: basal (BAP) and squeeze (SAP) pressure

<table>
<thead>
<tr>
<th></th>
<th>BAP (mmHg)</th>
<th>SAP (mmHg)</th>
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<tbody>
<tr>
<td>Before FI</td>
<td>28.8+6.8</td>
<td>97.6+16.2</td>
</tr>
<tr>
<td>After FI</td>
<td>0</td>
<td>12.4+2.2</td>
</tr>
<tr>
<td>Before RF</td>
<td>3.2+0.8</td>
<td>10.2+1.8</td>
</tr>
<tr>
<td>After RF</td>
<td>18.8+4.2</td>
<td>76.0+12.4</td>
</tr>
<tr>
<td>Before sacrifice</td>
<td>22.8+3.6</td>
<td>74.8+10.8</td>
</tr>
</tbody>
</table>

FI=faecal incontinence; RF=radiofrequency; BAP=basal anal pressure; SAP=squeeze anal pressure

Figure 1. Diagram of anal sphincter sections: PND=pudendal nerve destruction; LS=lateral sphincterotomy; AS=anal stretch; A-B=the part of the anal sphincters excised and divided (4X) for histopathological evaluation (10 samples from each part).
**Figure 2:** Low power haematoxylin and eosin (H&E) stain demonstrating a thinner muscularis propria in the UFI (left) compared with the FI+RF (right) (x100).
Figure 3: Increased collagen deposition within the septae (blue) in the UFI group (left) against the smooth muscle cells (red) compared with the FI+RF group (right) (Masson’s trichrome stain x100)
Figure 4:

Immunoperoxidase stain for CD117 showing a reduced number of ICC (arrows) in the FI+RF group compared with the UFI group (CD117 Cellmarque x1000)