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A Novel Animal Model for External Anal Sphincter Insufficiency

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Abstract:	<p>Purpose: Reliable animal models are essential to evaluate future therapeutic options like cell-based therapies for external anal sphincter insufficiency. The goal of our study was to describe the most reliable model for external sphincter muscle insufficiency by comparing three different methods to create sphincter muscle damage.</p> <p>Methods: In an experimental animal study female Lewis rats (200-250 g) were randomly assigned to three treatment groups (n=5, each group). The external sphincter muscle was weakened in the left dorsal quadrant by microsurgical excision, cryosurgery, or electrocoagulation by diathermy. Functional evaluation included in vivo measurements of resting pressure, spontaneous muscle contraction, and contraction in response to electrical stimulation of the afferent nerve at baseline and at 2, 4, and 6 weeks after sphincter injury. Mason trichrome staining and immunofluorescence for skeletal muscle markers was performed for morphological analysis.</p> <p>Results: Peak contraction after electrical stimulation was significantly decreased after sphincter injury in all groups. Contraction forces recovered partially after cryosurgery and electrocoagulation but not after microsurgical excision. Morphological analysis revealed an incomplete destruction of the external sphincter muscle in the cryosurgery and electrocoagulation groups compared to the microsurgery group.</p> <p>Conclusions: For the first time three different models of external sphincter muscle insufficiency were directly compared. The animal model using microsurgical sphincter destruction offers the highest level of consistency regarding tissue damage and sphincter insufficiency, and therefore represents the most reliable model to evaluate future therapeutic options. In addition this study represents a novel model to specifically test external sphincter muscle function.</p>
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Suggested Reviewers:	Klaus Matzel, Professor Friedrich Alexander University Erlangen-Nürnberg klaus.matzel@uk-erlangen.de Professor Klaus Matzel is internationally well known for his research and clinical expertise in the field of fecal incontinence. In particular he played a pioneering role in the development of sacral neuromodulation.

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A NOVEL ANIMAL MODEL FOR EXTERNAL ANAL SPHINCTER INSUFFICIENCY

Running title: Model for external anal sphincter insufficiency

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Abstract

Purpose: Reliable animal models are essential to evaluate future therapeutic options like cell-based therapies for external anal sphincter insufficiency. The goal of our study was to describe the most reliable model for external sphincter muscle insufficiency by comparing three different methods to create sphincter muscle damage.

Methods: In an experimental animal study female Lewis rats (200–250 g) were randomly assigned to three treatment groups (n=5, each group). The external sphincter muscle was weakened in the left dorsal quadrant by microsurgical excision, cryosurgery, or electrocoagulation by diathermy. Functional evaluation included in vivo measurements of resting pressure, spontaneous muscle contraction, and contraction in response to electrical stimulation of the afferent nerve at baseline and at 2, 4, and 6 weeks after sphincter injury. Mason trichrome staining and immunofluorescence for skeletal muscle markers was performed for morphological analysis.

Results: Peak contraction after electrical stimulation was significantly decreased after sphincter injury in all groups. Contraction forces recovered partially after cryosurgery and electrocoagulation but not after microsurgical excision. Morphological analysis revealed an incomplete destruction of the external sphincter muscle in the cryosurgery and electrocoagulation groups compared to the microsurgery group.

Conclusions: For the first time three different models of external sphincter muscle insufficiency were directly compared. The animal model using microsurgical sphincter destruction offers the highest level of consistency regarding tissue damage and sphincter insufficiency, and therefore represents the most reliable model to evaluate future therapeutic options. In addition this study represents a novel model to specifically test external sphincter muscle function.

Key words: anal incontinence, animal model, external sphincter, microsurgery

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4 **Introduction**
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6 Fecal incontinence limits daily activities and strongly influences the patient's
7 psychological, emotional, and social wellbeing.[1,2] The high prevalence[3,4,2] and the
8 significant socio-economic impact of the disease on one hand[5] and the limitations of
9 current therapeutic options especially for large defects of the external sphincter on the
10 other hand highlight the importance of ongoing research in the field.[6-8]
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20 Research concentrates on the external sphincter for several reasons: This part of the
21 sphincter muscle is much more voluminous in humans in comparison to the internal
22 sphincter muscle and is frequently affected after obstetric traumas.[9,10] The external
23 sphincter muscle is relevant in preventing urge incontinence[11,12] and contributes to the
24 maintenance of passive continence.[13]
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33 However, in previous studies, a variety of methods including sphincterotomy[14,15],
34 resection of the internal and external sphincter[16], electrocoagulation[17] or cryo-
35 injury[18] to generate models for sphincter muscle insufficiency have been used. Therefore
36 data comparison across the literature is difficult.
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44 Beside creation of a distinct defect of the external sphincter this muscle has to be
45 accessible to functional evaluation in order to test new therapeutic procedures. In vivo
46 measurement of the resting pressure has been described previously.[19,15] However, since
47 the resting pressure is generated by the internal *and* external sphincter[13] this examination
48 allows no individual functional evaluation of both muscles. A rat animal model that
49 permits assessment of squeeze pressure to specifically evaluate the external sphincter
50 muscle does not exist so far.
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1 This study was designed to describe a rodent animal model with a consistent, isolated
2 defect of the external sphincter, which offers functional *in vivo* evaluation of this muscle.
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4 We therefore compared three different techniques of isolated and irreversible external
5 sphincter injury including microsurgical excision, which has not been described before,
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7 cryosurgery and electrocoagulation.
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2 **Materials and methods**
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5 *Study design and protocol*
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9 The study was performed with the approval of the Animal Care Committee of the Canton
10 of Zurich, Switzerland. Preliminary experiments were conducted to determine the optimal
11 settings for the in vivo assessment of sphincter function. These experiments included
12 evaluation of the most precise localization for electrical nerve stimulation to induce
13 external sphincter muscle contraction.
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22 For the final evaluation, a total of 15 adult female Lewis rats (200–250 g) were randomly
23 placed into three groups (n=5, each group) for functional analysis. Another 6 animals
24 served for morphological investigations. Baseline manometric assessment included resting
25 pressure, spontaneous contractions, and contractions in response to electrical stimulation.
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27 As shown in Figure 1, the left dorsal quadrant of the external sphincter muscle was
28 microsurgically removed (MS group) or destroyed by cryosurgery (CS group) or
29 electrocoagulation using diathermy (EC group). Manometric evaluation was repeated at 2,
30 4, and 6 weeks after sphincter damage. Additionally, after 2 and 4 weeks one animal and
31 after 6 weeks four animals per group were sacrificed for tissue retrieval and histological
32 analysis. Samples from the untreated right-dorsal quadrant served as internal controls.
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51 *Animal anesthesia and surgery*
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54 All animals received Caprofen (Rimadyl®, Pfizer AG, Zürich, Switzerland; 5mg/kg
55 bodyweight) and Buprenorphine (Temgesic®, Reckitt Benckiser AG, Wallisellen,
56 Switzerland; 0.01-0.05 mg/kg bodyweight) by subcutaneous injection 30 minutes prior to
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surgery. Anesthesia was induced by inhalation of 5% isoflurane and maintained using 1–2% isoflurane. The surgical area was shaved and disinfected with povidone-iodine solution. After a left circum-anal incision the fat was separated from the lower rectum and anal canal under an operating microscope and the left dorso-lateral quadrant of the sphincter muscle was exposed.

For partial sphincter damage, approximately 25% of the circumferential external sphincter muscle fibers were removed or destroyed. Before microsurgical excision the external sphincter muscle was separated from the underlying internal sphincter muscle. Cryosurgery of the anal sphincter was induced in anesthetized rats using an aluminum rod measuring 2×5 mm, which was chilled in liquid nitrogen as was previously described.[18] The chilled probe was held towards the anal sphincter for 15 seconds. Sphincter injury by electrocoagulation was induced with the use of an electrical surgical bipolar forceps, similar to a technique used for urinary sphincter injury.[17] Electrocoagulation of the left-dorsal external sphincter muscle was performed on an area of 5×2 mm for 1 second. Resorbable suture material was used for skin closure.

After surgery observations were made every 15 minutes until the animals were fully awake and active. Thereafter, animals were returned to their normal housing. Caprofen (5 mg/kg bodyweight once daily) and Buprenorphine (0.01–0.05 mg/kg bodyweight twice daily) were administered subcutaneously for 48 hours. Animals were carefully monitored for signs of infection.

Anal manometric evaluation

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3 All examinations were done under general anesthesia in the supine position. Based on
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5 previous studies[19,15] we used a saline-filled latex balloon connected through PE-100
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7 tubing to a pressure transducer (SP 844, Memscap, Crolles Cedex, France) and a digital
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9 data acquisition system (Powerlab 4/35 with LabChart Pro, ADInstruments, Spechbach,
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11 Germany) for anal manometric measurements. After the balloon was inflated to 10 cm
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13 H₂O and pressure calibration of the system was accomplished. After adaption of 15 to 30
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15 minutes, spontaneous contraction waves were recorded for 5 minutes. The peak
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17 contraction, area under the curve (AUC), and frequency of the contraction waves were
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19 recorded (Fig. 2A). To determine the resting pressure, the balloon probe was drawn out in
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21 a contraction interval and the resulting difference to the baseline pressure (mean of 5
22
23 intervals) calculated. For the assessment of sphincter contraction after electrical
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25 stimulation of the inferior rectal nerve, a bipolar electrode was inserted through a 2-mm
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27 incision at the ventral verge of the anus. We used a Grass S48 Stimulator (Grass
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29 Technologies, Astro-Med, Rodgau, Germany) at 30 Hz (pulse duration 1ms), 30 V for 2
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31 seconds for the electrical stimulation. The best stimulation location was defined as the
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33 place with minimal leg twitching and maximal sphincter response. The peak contraction
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35 force and the maximal slope of the contraction waves were registered (Figure 2B). An
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37 average of five contractions was used for statistical analysis.
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Histology and immunohistochemistry

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55 Animals were sacrificed under anesthesia by thoracotomy and incision of the heart. After a
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57 circum-anal incision the anus and lower rectum were removed en-bloc. The total specimen
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1 was fixed in formalin for 24 hours and rinsed in phosphate buffered saline. After
2 dehydrating overnight in a histokinette (Tissue Processor TP 1020, Leica Microsystems,
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4 Wetzlar, Germany) the samples were embedded in paraffin.
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8 The blocks were sectioned into 3- μ m slices using a microtome (Model RM 2155, Leica
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10 Microsystems, Wetzlar, Germany). The sections were fixed and labeled with Mason's
11
12 trichrome stain. Sphincter muscle cells were identified via immunocytochemistry using an
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14 anti- α -actinin antibody (Sigma-Aldrich, Buchs, Switzerland) at a dilution of 1:100. Native
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16 sphincter tissue sections served as positive controls. Tissue sections without primary
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18 antibody were used as negative controls. Alpha-actinin labeled cells were identified by
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20 fluorescence microscopy (Leica DM 6000 B / DFC 350 FX, Leica Microsystems, Wetzlar,
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22 Germany) using an excitation wavelength of 550 nm. Nuclei were stained with DAPI (4',6-
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24 diamidino-2-phenylindole; Sigma-Aldrich, Buchs, Switzerland). Image analysis to
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26 determine signal intensity per high power field was performed (ImageJ 1.45s, Wayne
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28 Rasband, National Institutes of Health, Bethesda MD, USA).
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39 *Statistical analysis*

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42 All experimental groups contained five animals for functional analysis at each time-point.
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44 The presented data are expressed as averages \pm the corresponding standard error of the
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46 mean. The repeated measures analysis of variance (ANOVA) was performed to assess the
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48 group effect for quantitative variables. The Bonferroni pairwise multiple comparison test
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50 was used to compare measurements after the intervention with the baseline values within
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52 the groups (time effect). Measurements at the end of the experiment were compared with
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54 unpaired students t-tests. All tests were performed bilaterally with a 5% significance
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threshold. The NCSS/PASS software package (NCSS, Kaysville, UT, USA) was used for
all calculations.

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Results

No intraoperative or postoperative complications occurred in either group. In particular, no rectal perforation, anal stricture or wound infection occurred.

Manometric evaluation during electrical nerve stimulation

Peak contractions were significantly reduced in all groups after model generation when compared to normal pressures (MS: 6.7 ± 1.9 cmH₂O vs 25.7 ± 2.1 cmH₂O; EC: 7.9 ± 1.8 cmH₂O vs 26.2 ± 2.8 cmH₂O; CS: 6.6 ± 1.8 cmH₂O vs 26.5 ± 0.7 cmH₂O). Peak contraction pressures were only reduced after cryosurgery and microsurgery over all time-points investigated as is demonstrated in Figure 3A. However, the values after microsurgery were significantly lower. The slope of the contraction waves recovered over time after sphincter damage by cryosurgery and electrocoagulation (Fig. 3B). The values in the microsurgery group were significantly lower for peak pressures (MS: 6.2 ± 1.1 cm H₂O; EC 13.8 ± 2.2 cm H₂O; CS 15.8 ± 2.2 cm H₂O) and slope of the contraction waves (MS: 18.4 ± 2.5 cm H₂O/sec; EC 33.5 ± 7.4 cm H₂O/sec; CS 39.2 ± 5.4 cm H₂O/sec) compared to the other groups at the end of the experiment (6 weeks after model generation).

Spontaneous manometric evaluation

Spontaneous, rhythmic contraction waves of the sphincter muscles demonstrated great variability between time-points and animals (Fig. 4). Peak contraction, area under the

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curve (impulse), frequency, and resting pressures did not differ from baseline values or between the groups. Resting pressures demonstrated a trend toward temporary recovery after 2 and 4 weeks, respectively (Fig. 4D).

Histology and immunohistochemistry

The external sphincter muscle was consistently removed at all time points after microsurgery (Fig. 5A-D). After cryosurgery and diathermy skeletal musculature from the external sphincter muscle was partially recognizable at all time points (Fig. 5F-H and J-L). No significant inflammatory reaction was registered at later time points.

A sustained loss of muscle tissue in the MS group (28.0 ± 4.9 pixels $\times 10^3$ /high power field 6 weeks after model generation) compared to the control group (462.9 ± 28.4 pixels $\times 10^3$ /high power field) was demonstrated in immunofluorescence analysis using α -actinin as a specific musculoskeletal marker (Fig. 6B-D, M). Reduction of α -actinin expression was less pronounced in the EC and CS groups, showing values of 143.4 ± 8.3 and 204.8 ± 9.2 pixels $\times 10^3$ /high power field, respectively (Fig. 6H, L, M).

Discussion

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3 In this study, three rat animal models for the evaluation of the external anal sphincter
4 function have been explored. The results from this study objectively reveal functional and
5 morphological regeneration after cryosurgery and electrocoagulation after 6 weeks, which
6 was not found after microsurgical excision.
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13 The advantage of microsurgery seems to be that skeletal muscle tissue can be removed
14 under direct visual control until no muscle fibers are left. On the other hand, after
15 cryosurgery and electrocoagulation necrotic tissue remains, and the depth of the injury
16 cannot be precisely controlled. Thus, potentially vital skeletal muscle fibers remain
17 underneath or in the necrotic area and repair mechanisms based on satellite cells might
18 help to quickly regenerate muscular tissue.[20] In order to prevent such a mechanism the
19 interval and intensity of cryosurgery or electrocoagulation must be increased. However,
20 individual anatomical differences and lacking possibilities to control the depth of
21 penetration increase the risk to go beyond the target by destroying the adjacent internal
22 sphincter muscle.
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39 We did not observe any changes at the different time points or between models after either
40 method of external sphincter destruction for frequency, peak force, and impulse (area
41 under curve) of spontaneous contraction waves in the anal canal. These results support the
42 current understanding that these spontaneous contraction waves are provoked by
43 contractions of the internal sphincter,[13] which was not intended to be damaged in our
44 model. Involvement of the internal sphincter using cryosurgery and electrocoagulation is
45 possible since the depth of destruction with these methods is more difficult to control.
46 Therefore differences between models during spontaneous contractions may be detected
47 with a larger sample size. However, since our model was developed for external sphincter
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assessment our experiments were not designed to find differences in spontaneous contractions, which are related to internal sphincter function.

After destruction of the external sphincter a significant impairment of the resting pressure would be expected.[13] Conversely, only a non-significant reduction of sphincter pressure could be demonstrated in all groups. This might be due to a distinct anatomical difference between humans and rats, among which the external sphincter is less voluminous. With regard to the resting pressure, it seems that in rats the internal sphincter can compensate the damaged external sphincter. Moreover, a transient trend towards recovery of the resting pressure with a peak after 2 and 4 weeks might be explained by postoperative edema.

Indeed, the ratio of thickness between internal and external sphincter muscle in rats is not exactly comparable to humans. This fact does not signify a limitation of the present study since this model was specifically developed to study damage of the external sphincter muscle. This muscle can be selectively assessed for functional and morphological analysis, which underlines the relevance of this model. Selective assessment of the external sphincter has been described in larger animals[21], such as rabbits or dogs. Compared to these experiments our rat model seems to provide similar possibilities of external sphincter evaluation in the lowest possible species. One of the main restrictions of our model is the translation of the resting pressure measurements to humans. However, resting pressure is always a product of internal *and* to a minor part of external sphincter muscle function[13] and is therefore not suitable for external sphincter assessment.

A limitation of this study might further be represented by the very critical exact position of the probe for electrical stimulation of the afferent nerve for muscle contraction. In order to encounter this potential bias, the best stimulation location was defined as the place with

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2 minimal leg twitching and maximal sphincter response. An average of five contractions
3 was then used for statistical analysis.
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6 Our study has a number of important strengths. First, a reliable animal model facilitates
7 both evaluation of novel therapy options and physiological studies of underlying
8 mechanisms. By comparing different methods of sphincter destruction this study represents
9 an important landmark to encounter efforts for standardization and rationalization of
10 animal models and evaluation methods for studying the anal sphincter.
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19 Second, with regard to testing of future therapy options including cell-based
20 therapies[22,18,14] or tissue engineering[23] for external sphincter muscle regeneration we
21 chose to induce sustained long-term external sphincter muscle damage. Previous studies
22 evaluating such therapies were based on animal models with destruction of both sphincter
23 muscles.[22,18,14] Still, in such a model function of one muscle might be influenced by
24 the destruction of the other muscle, which complicates interpretation of outcome measures.
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34 Our animal model presented in this study consists of an isolated defect of the external
35 sphincter and will therefore allow unbiased evaluation of this muscle.
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41 Third, with this study we describe a method to selectively assess external sphincter
42 function of rats *in vivo* by electrical stimulation of the inferior rectal nerve and balloon
43 manometry. Functional evaluation of the external sphincter muscle was performed so far
44 *ex vivo* using organ bath studies.[18,14]. However, conclusions from a denervated piece of
45 muscle, which is isolated from its surroundings on *in vivo* function, have consequently to
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1 contraction force. For that purpose a novel method to specifically assess external sphincter
2 function in rats was described. The objective analysis of three distinct methods for creating
3 a model of external sphincter muscle insufficiency provides evidence that sphincter
4 destruction by microsurgery represents the most reliable model in terms of persisting
5 morphological and functional deficiency compared to cryosurgery and electrocoagulation.
6
7 Therefore, this animal model should be considered when evaluating novel therapeutic
8 options for external sphincter muscle defects.
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Figure legends

Figure 1

Model generation: A cross-section of the anal canal in Mason trichrome staining. The dorsal-left quadrant of the external sphincter muscle was resected by microsurgery (between arrowheads). The internal sphincter muscle (arrow) was left intact.

Figure 2

(A) Representative example of spontaneous contractions of the sphincter muscles registered by balloon manometry in a normal animal. (B) Representative example of contractions of the sphincter muscles after electrical stimulation of the branches of the inferior rectal nerve registered by balloon manometry.

Figure 3

Differences between models (repeated measures ANOVA) for contraction (A) and maximal slope of the contraction wave (B) demonstrating the most reliable and long-term pressure reduction after microsurgery. Asterisks indicate a significant difference from baseline value. Values are given as means \pm standard error of the mean.

Figure 4

Comparing spontaneous contractions measured by balloon manometry: Peak forces (A), area under the curve (B), frequency of spontaneous contractions (C), and resting pressures (D) were subject to great variability and did not differ from baseline or between treatment groups. A trend towards a temporary recovery of the resting pressure was shown in all groups (D).

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Figure 5

Histomorphological assessment at three time points (A-L). Mason's trichrome staining showing smooth muscle cells in purple-red, skeletal muscle cells in red, and extracellular matrix in blue. The predominance of skeletal muscle to extracellular matrix at all postoperative time points is higher after model generation by electrocoagulation (F-H) or cryosurgery (J-L).

Figure 6

Cross sections of the anal canal at the level of the sphincter injury in the different groups are shown at different time points (A-L). Immunostaining (red) with the skeletal muscle marker (α -actinin) demonstrates persistence and/or recovery of skeletal muscle in the damaged area after cryosurgery or electrocoagulation. Nuclear counterstaining was done with DAPI. (M) Intensity (n=4) of the signal for immunofluorescence of α -actinin 6 weeks after model generation (pixels per high power field). Error bars indicate standard error of the mean.

Figure 1
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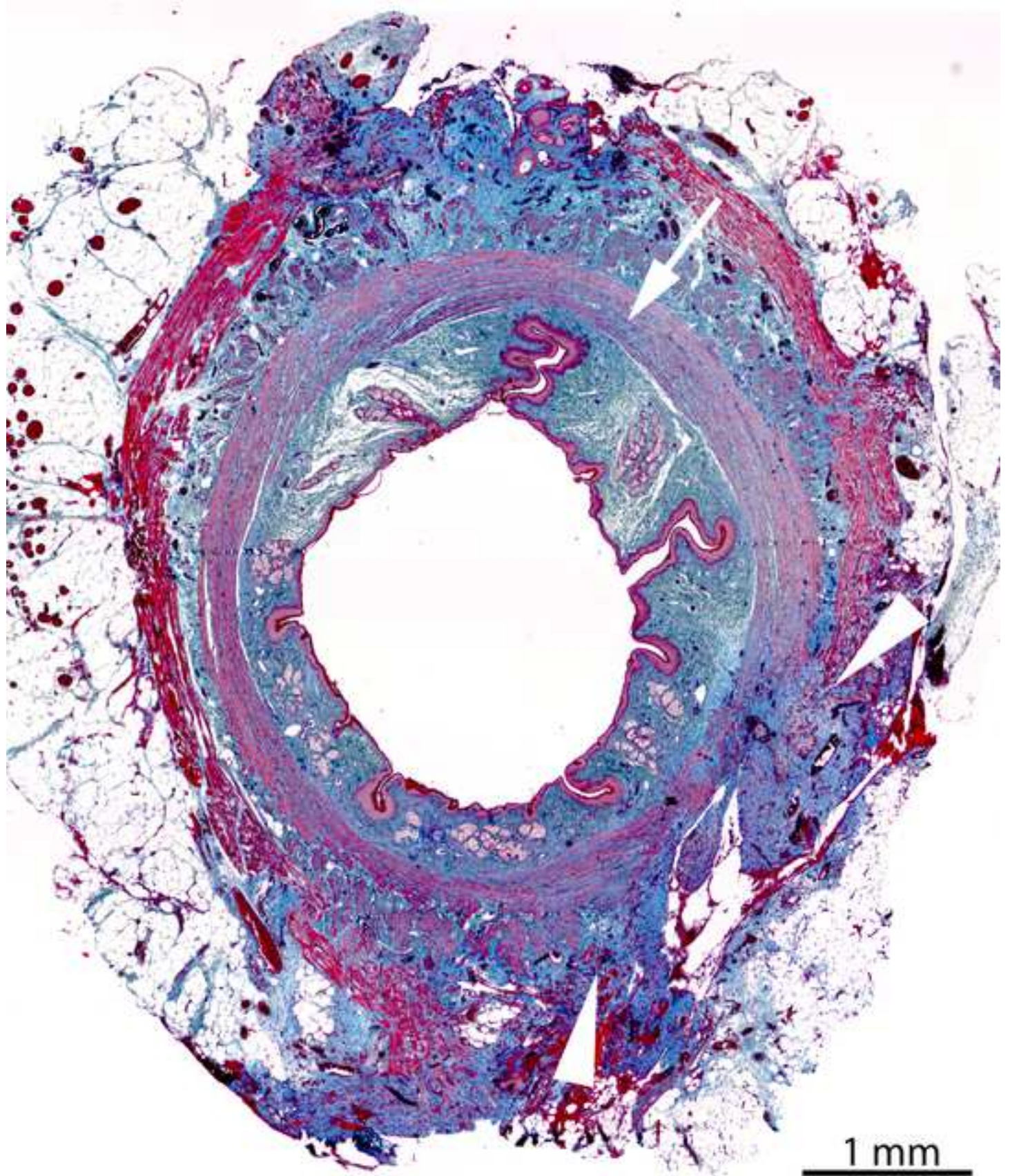
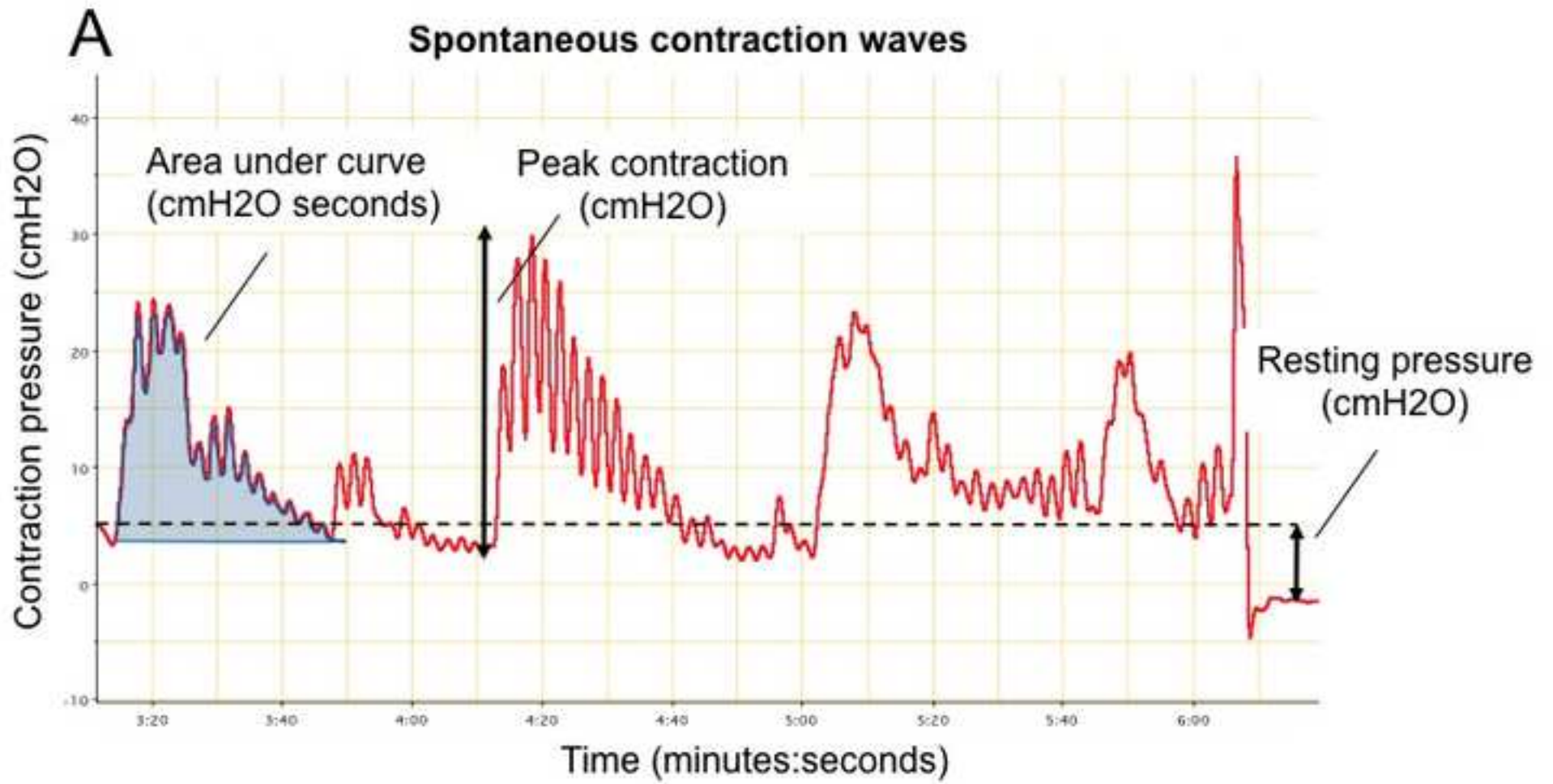


Figure 2a
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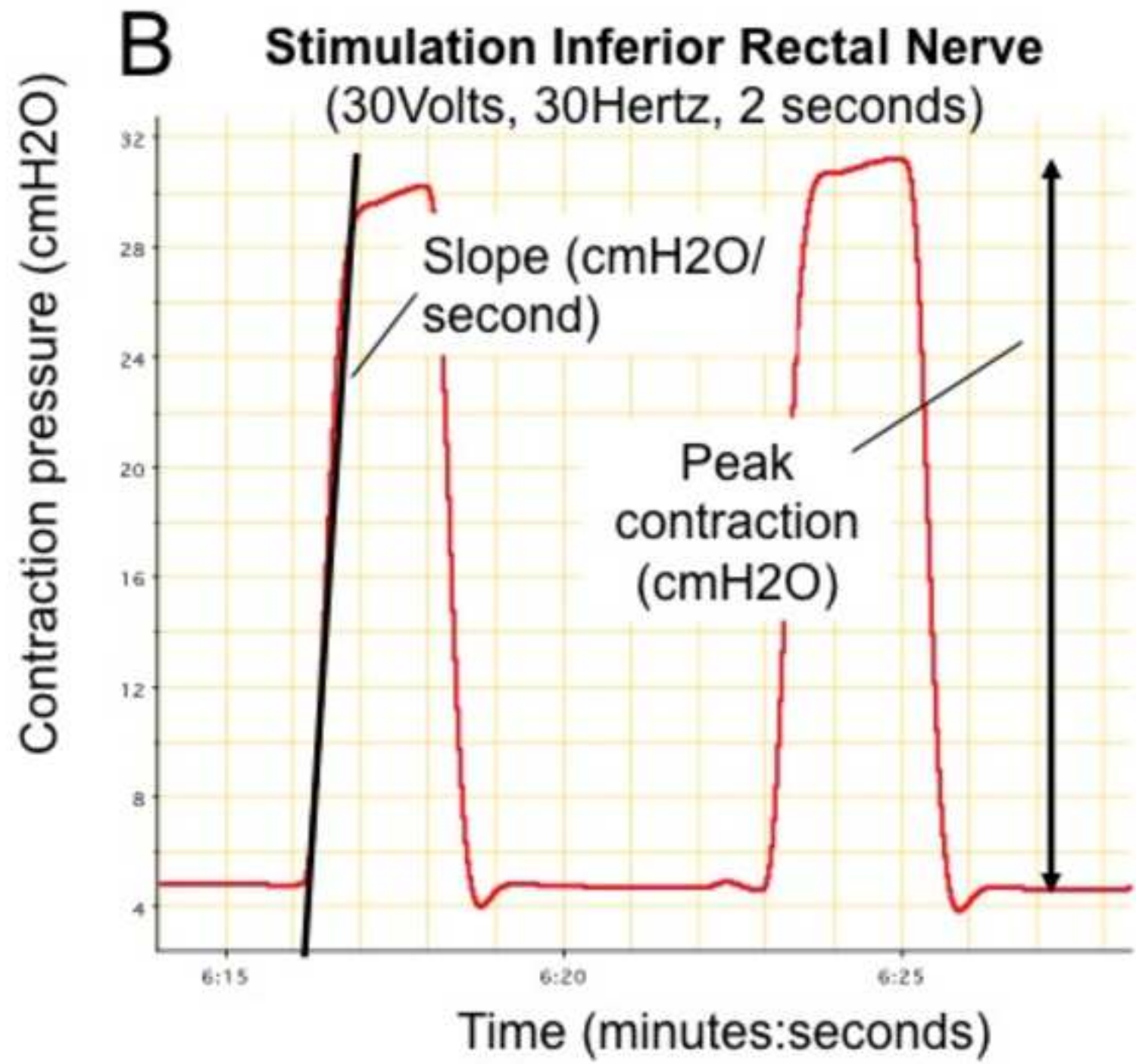


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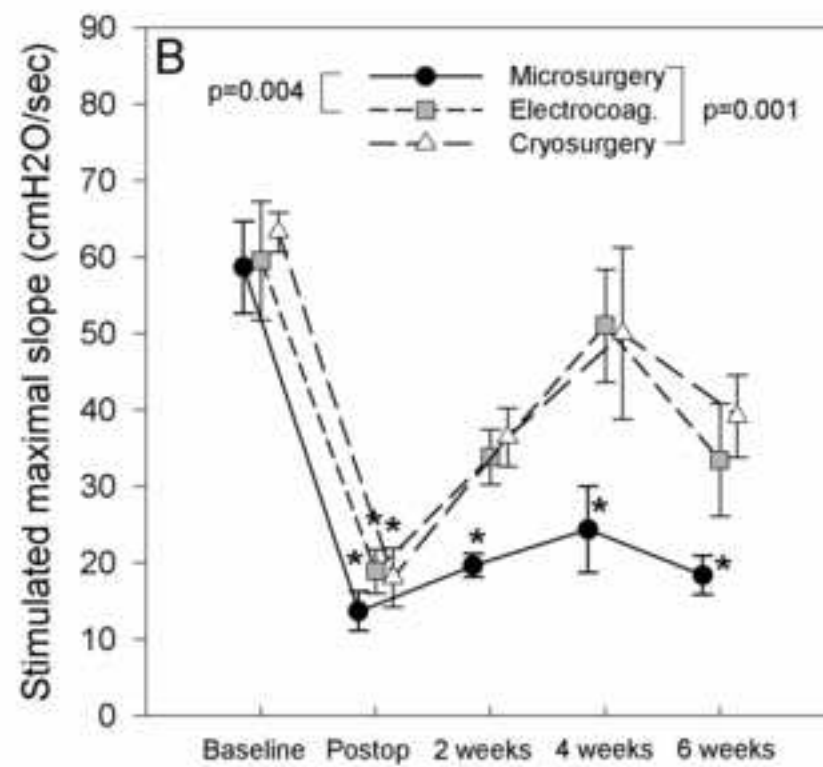
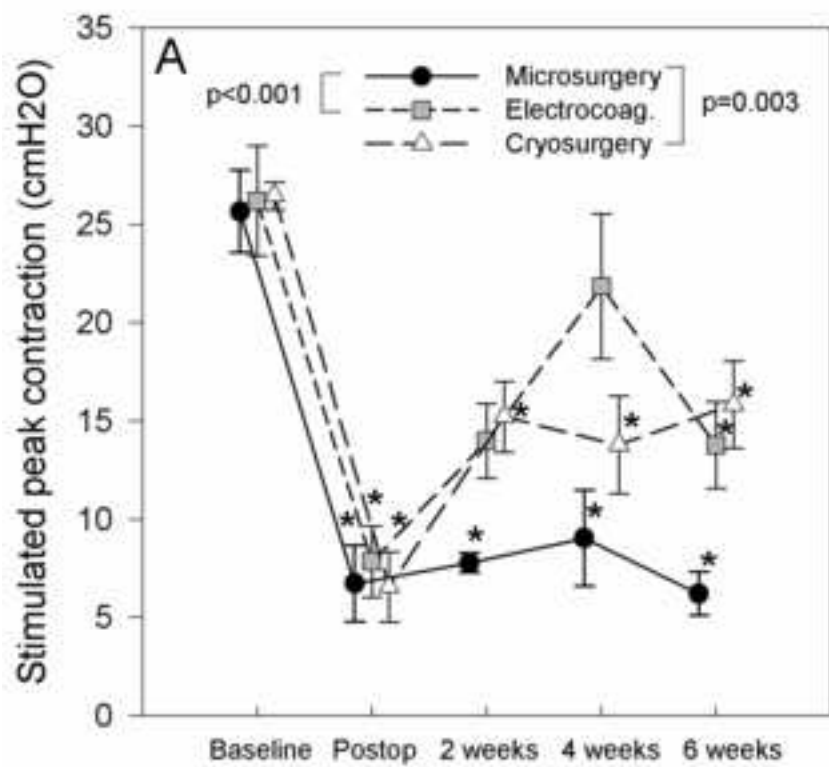


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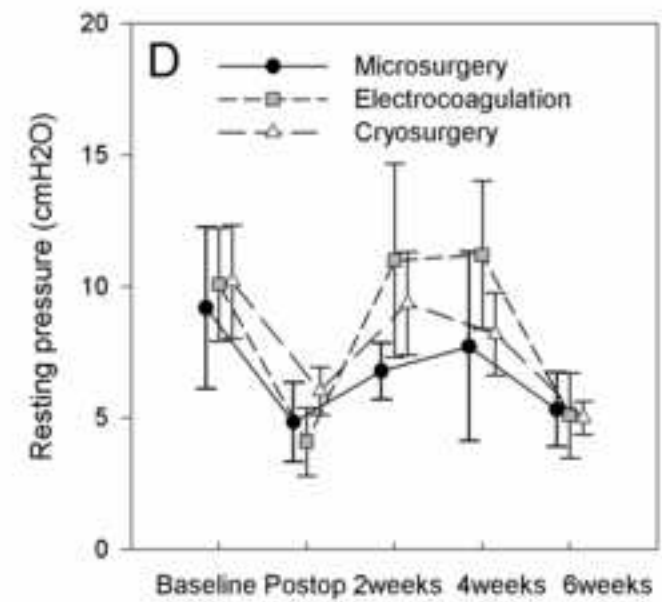
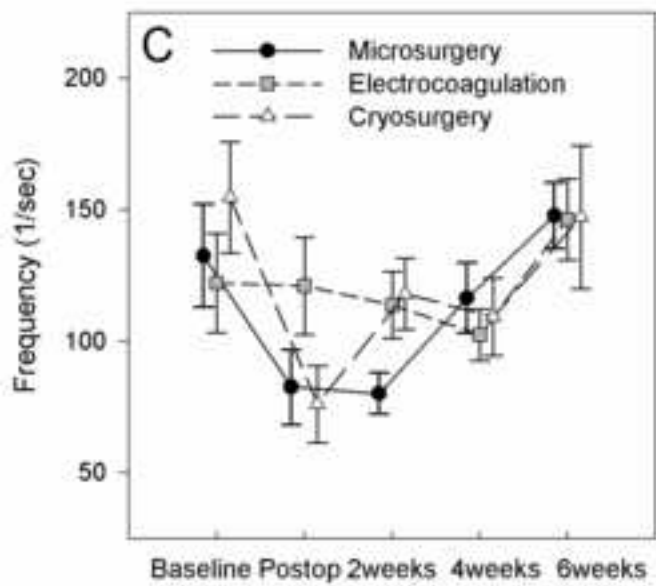
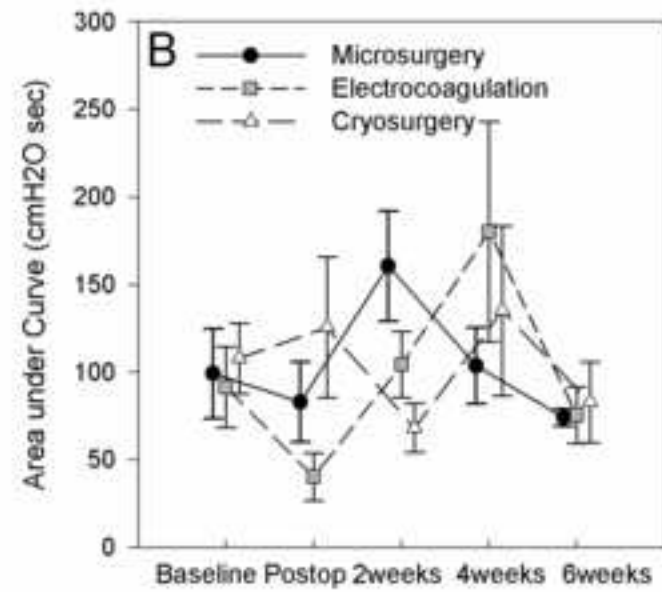
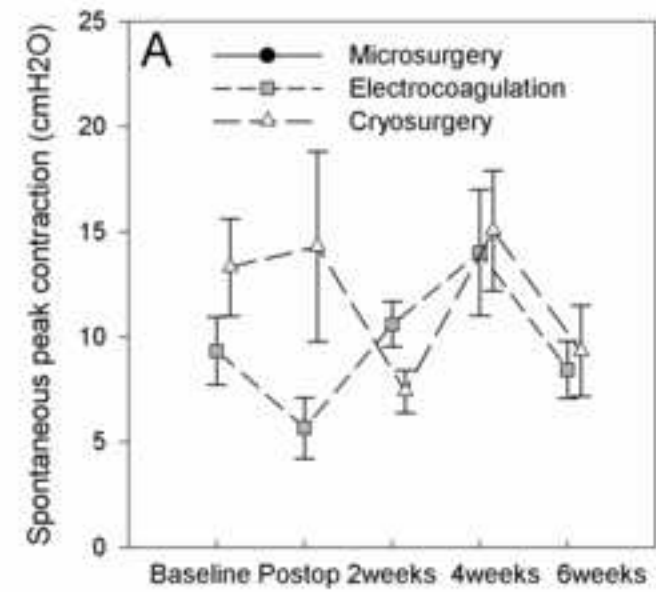


Figure 5
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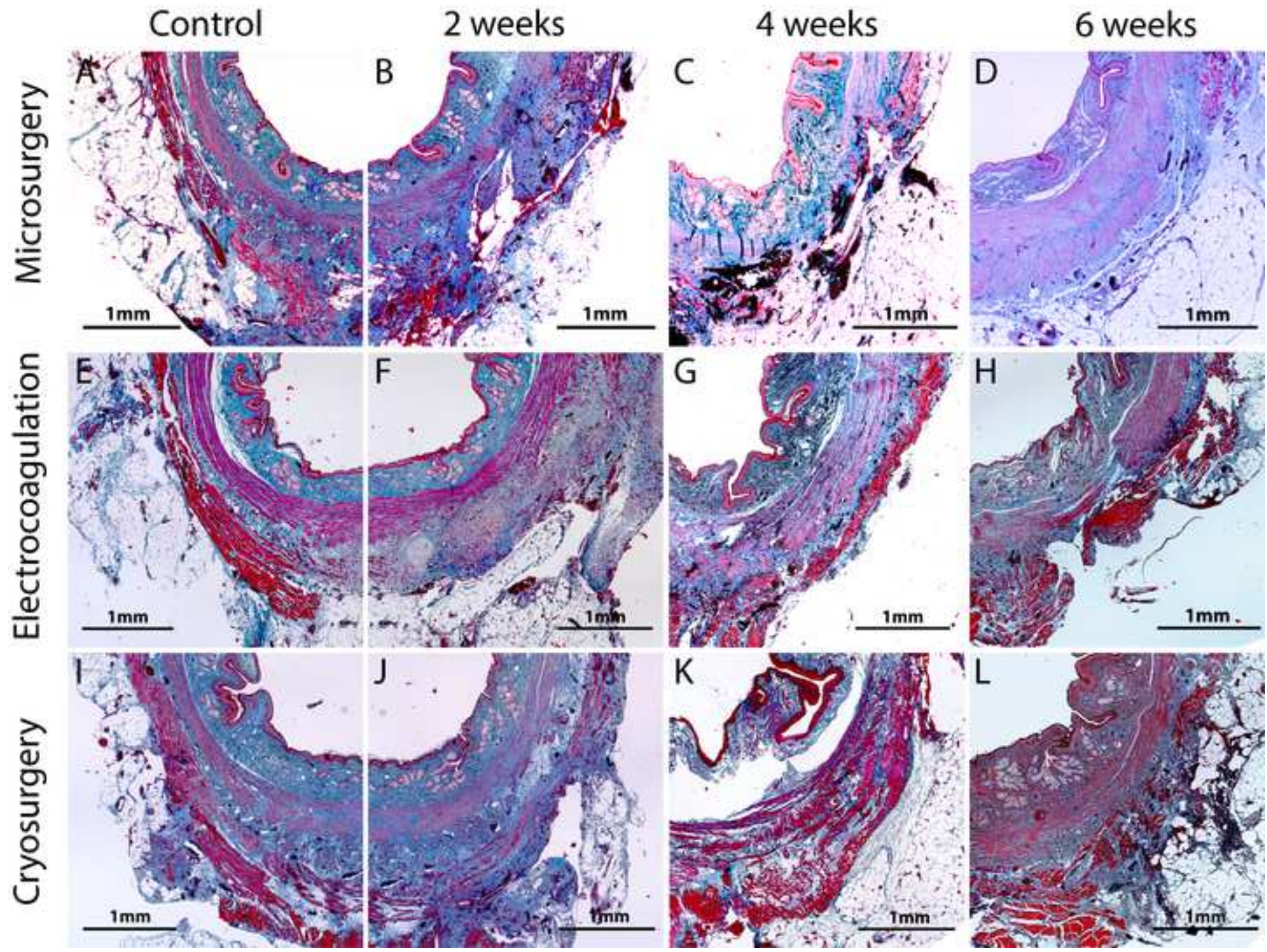


Figure 6

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