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Title:

RESIDUAL SUBSTANCE P LEVELS AFTER CAPSAICIN TREATMENT CORRELATE WITH TENDON REPAIR

Running head:

SP LEVELS AFTER CAPSAICIN TREATMENT ARE LINKED TO HEALING

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ABSTRACT

The aim of the study was to assess healing after capsaicin-induced SP depletion during rat Achilles tendon repair by biomechanical testing.

Capsaicin treatment reduced the concentrations of substance P (SP) by ~60% and calcitonin gene related peptide (CGRP) by ~40% as compared to the control group, as assessed by radioimmunoassay in the dorsal root ganglia (DRG), at 1 and 4 weeks post tendon rupture. Also the peripheral neuronal presence of SP and CGRP, as assessed by immunohistochemistry, was lower at both weeks 1 and 4. The decreased peripheral neuronal presence of SP at week 1 correlated with the corresponding levels in the DRG ($r=0.54$, $p=0.018$). The reduced presence of SP/CGRP after capsaicin treatment was verified by a decreased sensitivity to painful mechanical and thermal stimuli ($p< 0.05$).

Correlation analyses between individual residual SP levels and biomechanical tissue properties were performed due to differences in failure mode between the groups and high individual variations in the SP levels after capsaicin treatment. Thus, the residual SP levels in the DRG correlated with transverse area, ultimate tensile strength and stress at failure ($r=0.39$, $p=0.036$; $r=0.53$, $p=0.005$ and $r=0.43$, $p=0.023$, respectively). Furthermore, individual pain sensitivity at week 2 correlated with peripheral occurrence of SP and was correlated with tensile strength and stress at failure ($r=0.89$, $p=0.006$ and $r=0.78$, $p=0.015$) at 4 weeks.

In conclusion, rats with higher residual SP levels after capsaicin-induced neuropathy develop improved tensile strength and stress at failure in the healing Achilles tendon.

Key Words: Achilles tendon, chemical sensory denervation, healing, sensory neuropeptides, biomechanical properties

INTRODUCTION

Tendon repair is characterized by prolonged healing and rehabilitation compared to that of many other tissues, and often with a suboptimal outcome (1, 2). Deficient tendon repair is especially marked in patients with neuropathic conditions such as diabetic neuropathy (3, 4). Hence, the scarce innervation in normal tendons (5) in contrast to other tissues might partly explain the prolonged healing process, but also its potential importance in normal healing in this tissue.

A role for neuropeptides in the healing of other tissues such as skin (6), bone (7), ligaments (8), and cornea (9, 10) has been documented in the literature. In skin, this effect has been documented by adding exogenous neuropeptides such as Substance P or Calcitonin Gene Related Peptide (CGRP) to deficient wound healing sites (11, 12), as well as in diabetic animals (13-15). Thus, neural influences on wound healing appear to be a general function for optimal healing.

Accumulating data also suggest that a variety of neuronal mediators, neuropeptides, in addition to cytokines and growth factors play an important role in the tendon repair process (16-20). Thus, the sensory neuropeptides, substance P (SP) and calcitonin gene related peptide (CGRP), have been shown to exert stimulatory effects during angiogenesis and proliferation of several cell types active during tissue repair (21-24).

After rupture, healing tendons present with a pronounced ingrowth of nerves, which exhibit a specific time-dependent expression for SP and CGRP during the inflammatory, regenerative and remodelling phases of healing (25, 26). This process is paralleled with a concurrent expression of receptors for SP and CGRP in the healing area (16). Recent studies have demonstrated that intralesional administration of SP after ligament injury and Achilles tendon rupture induces an increased occurrence of fibroblasts, an accelerated collagen fibre alignment, and also led to improved biomechanical properties of the healing tissue (8, 17, 19).

Given that sensory neuropeptides exert trophic effects, the impaired healing in different neuropathic conditions may be a result of a reduced local presence of sensory neuropeptides. Hence, the aim of the present study was to investigate whether decreased concentrations of SP and CGRP during Achilles tendon healing would influence the development of biomechanical tissue properties.

MATERIALS AND METHODS

The study included 85 male Sprague Dawley rats with an initial weight of 115-130 grams housed three to four per cage at 21°C in a 12:12h light:dark cycle with water and pellets *ad lib*, according to the Karolinska Institutets protocol. The experiments were approved by the local animal ethics committee.

Denervation: All 85 rats (Fig. 1) were anaesthetized by Forene® inhalation (Abbot Scandinavia AB, Kista, Sweden) 4% in O_2, 2 l/min. In order to induce a chemical sensory denervation, 45 rats (*T-group*) were injected with 50 mg/kg capsaicin (Sigma, St. Louis, USA) subcutaneously on the dorsal torso in a vehicle of 10% ethanol and 10% Tween 80 in isotonic saline for 4 consecutive days. The neurotoxin capsaicin, in the doses used, has been demonstrated to induce up to 50% depletion of sensory neuropeptides (SP and CGRP) in primary sensory neurons (27). The remaining forty rats (*C-groups*) served as denervation controls and were subsequently injected with corresponding amounts of vehicle. To reduce respiratory symptoms, the animals were injected with 15mg/kg theophylline (ACO, Stockholm, Sweden) 15 minutes prior to each injection of capsaicin or vehicle. Injections were completed 7 days before induction of Achilles tendon rupture (see below). Sixteen rats were lost after the first day of treatment with capsaicin, a percentage similar to previous studies (28).

Verification of denervation: The denervation process was evaluated through a clinical test as well as direct measurements of the concentration of sensory neuropeptides. Measurements of hindpaw withdrawal latencies (see below) were performed in order to identify the functional influence of the capsaicin treatment on the sensory nervous system. Radioimmunoassays and immunohistochemical analyzes (see below) were performed in order to quantify the effects on peripheral, as well as on central levels of sensory neuropeptides.

Surgery: Fifty-nine rats (29 denervated (*T-group*) and 30 denervation controls (*C-group*)), were anaesthetized by Forene® inhalation (Abbot Scandinavia AB, Kista, Sweden) 3-4% in O_2, 1-2 l/min. A 1-cm longitudinal midline incision was made directly above the right Achilles tendon. The incision penetrated the skin and crural fascia exposing the proper Achilles and plantaris tendons. The Achilles tendon was ruptured in the middle, i.e. approximately 0,5cm from the calcaneal insertion and the musculotendinous junction, respectively, using a blunt instrument tearing the tendon fibers apart. This method was chosen to mimic the clinical situation where the Achilles tendon faces a sudden overload when ruptured. The tendon was left unsutured, while the plantaris tendon was left intact, serving as an internal support. The skin was sutured with 5/0 non-resorbable nylon (Ethilon® II, Ethicon, Sommerville, NJ, USA). An additional two rats were lost during subsequent anaesthesia and surgery.

In the remaining 10 non-operated control rats (*C-0w*), the Achilles tendons were left intact. All surgical procedures were performed under sterile conditions. All animals were allowed free postoperative cage activity.

Nociception: The hind paw withdrawal latencies (HWLs) following thermal and mechanical stimulation were measured after denervation just prior to surgery. Thereafter, the HWLs were measured at 1, 2, 4, 6 and 8 weeks post surgery. The Randall Selitto Test (Ugo Basile, Type 7200, Italy) was used to assess the HWLs to mechanical stimulation. The rat hind paws were, one at a time, placed on an even and elevated surface while a wedge-shaped pusher put an increasing load on the dorsal surface. The loading rate was 30 g/s. The latency required to evoke a withdrawal response was measured and expressed in seconds. The Plantar Test (Ugo Basile, Type 7371, Italy), as described previously (29), was used to assess the HWL to thermal stimulation. In short, the plantar surface of the rat hind paws was placed on a transparent surface, one paw at a time. Underneath, an infra red light beam was applied,

corresponding to 52ºC. The time to hind paw withdrawal was measured in seconds (s) and referred to as the HWL to thermal stimulation. By measuring the mechanical and thermal HWL, after denervation but before tendon disruption, a reference value (baseline) was obtained. The average HWL for the hind paws was subsequently calculated. The longitudinal development of the mechanical and thermal HWL was assessed by repeated measurements of the right hindpaw.

Dissection and tissue harvest: The *T-* and the *C-group* were each randomly divided into three different subgroups of equal size and subsequently animals in the subgroups were euthanized at post operative week 1 *(T – 1w; C – 1w)*, 4 *(T – 4w; C – 4w)* and 8 *(T – 8w; C – 8w)*, respectively. The intact controls (*C-0w)* were euthanized at the end of the experiment. On the day of dissection, the rats were anaesthetized by an injection of Pentobarbitalnatrium® (60mg/ml, Apoteket, Sweden) (60mg/kg bw, i.p.), subsequently decapitated and exsanguinated. The right Achilles tendon including the posterior part of the calcaneus bone and the skin from the dorsal and plantar aspects of the right hind paw were dissected. From the central nervous system, the 2nd to 6th right dorsal root ganglia (*DRG*) and the spinal cord (*SC*) corresponding to this level, i.e. innervating the hind limb, were dissected. The Achilles tendons were frozen at -20ºC after dissection, while the DRG and SC samples were weighed immediately and snap frozen on dry ice. The DRGs from each animal were pooled as one sample. The tendons, DRG´s and SC were subsequently maintained at -70ºC until analyzed. The skin samples were immersed in Zamboni´s fixative consisting of 4% paraformaldehyde in 0.2 mol/l Sörensen phosphate buffer, pH 7.3, containing 0.2% picric acid for at least 2 h at room temperature. The samples were thereafter rinsed four times in PBS. The specimens were soaked in 20% sucrose in 0.1 mol/l Sörensen phosphate buffer, pH 7.2, containing sodium azide over night, and this procedure was repeated until all visible Zamboni fixative was removed. The samples were thereafter kept at +8ºC until analyzed.

Radioimmunoassay (RIA): The samples were extracted in 2 mol/l acetic acid, sonicated (60s) and then centrifuged at 3000g for 15 minutes. The supernatants were lyophilized and kept at -70°C for storage. Before the samples were analyzed, they were dissolved in 1ml of RIA buffer (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and subsequently diluted. After a dilution series was prepared and assessed, the amount of sample was set to 1/20 (SC), 1/1 (DRG) for SP and 1/160 (SC) and 1/10 (DRG) for the CGRP analyses. **SP-** and **CGRP**-LI were assessed using commercially available rabbit anti-rat SP and CGRP RIA kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The antibody for SP had 100% reactivity for rat substance P, while the antibody for CGRP had 100% reactivity for rat α-CGRP and 78% reactivity for rat β-CGRP. The assays were performed as recommended by the supplier. The detection limit was set to 8 pmol/l (IC50 = 166.6 pmol/l) for SP, and to 22 pmol/l (IC50 = 76.9 pmol/l) for CGRP. The intraassay coefficient of variation was 3.8% for SP and 5.5% for CGRP. The concentrations of SP and CGRP were expressed as pmol/g of total protein content (see below).

Protein determination: The protein concentrations of the homogenized RIA-samples were assessed according to Lowry.

Immunohistochemistry (IHC): The skin samples from the dorsal and plantar aspect of the right hindpaw were both placed on cryo blocks and sectioned at 15 μm on a Leitz cryostat. The sections were subsequently mounted directly on Super-Frost/Plus glass slides and immunostained. The slides were rinsed for 10 minutes in PBS. Incubation with 10% normal goat serum in PBS for 30 minutes blocked nonspecific binding. Sections were thereafter incubated with primary antisera for a marker of mature nerves, PGP-9.5 (1:10 000, UltraClone, Wellow, UK), as well as SP and CGRP (1:10000, Peninsula Laboratories, CA, USA) over night in a humid atmosphere at +8°C. After incubation with the primary antisera, the sections were incubated with biotinylated goat anti-rabbit antibodies (1:250, Vector

Laboratories, Burlingame CA, USA) for 40min at room temperature. Finally, the sections were incubated for 40min with Cy_2-conjugated avidin (1:2000, Amersham International plc, UK). Control staining was performed by omitting the primary antiserum. A preadsorbtion test was conducted previously using the same antibodies and tissues (31).

Morphology: A Nikon epifluorescence microscope (Eclipse E800 Yokohama, Japan) was used to analyze the tissue sections. The neuronal occurrence of perivascular and subcutaneous staining for SP and CGRP was verified by PGP-9.5 staining. The assessments were conducted by an experienced blinded observer. Pictures were taken with a video camera system (Nikon, digital camera DXM 1200, Japan) for later computerized analysis.

Computerized analyses: Semi-quantitative image analysis was used to assess the fractional area occupied by nerve fibers immunoreactive to anti-SP and anti-CGRP in relation to total area. This was done by a method described in detail elsewhere (25). In short, pictures were taken of the three areas with the highest occurrence of positive staining. The pictures were subsequently analyzed using the software Easy Image Analysis 2000© (Tekno Optik AB, Sweden). The image analysis algorithm takes the area as well as the intensity of the immunological staining into account. Earlier studies have determined the mean coefficient of variation for two observers to be 9.8% and the mean coefficient of variation for one observer to be 9.6% (25).

Biomechanical testing: Before testing, the Achilles tendon samples were removed from the -70ºC freezer, and defrosted wrapped in tissue paper soaked in 1% saline. The samples were kept moist using an airflow system that provided 99% relative humidity at 37ºC. This method of post mortem storage has been thoroughly studied and no significant effects on the parameters studied here have been shown (32). The proximal end of the tendon was gripped at the musculotendinous junction in a Peltier-effect cryoclamp (Tissue Grip, GRP-TE-2250N, EnduraTEC®, USA) mounted on an EnduraTEC 450N load cell (EnduraTEC®, USA) while

the distal end was gripped in a custom made clamp that held the calcaneal bone at an angle of 60º to allow the tendon fibers to be aligned in a perfect vertical position. The tissue was carefully aligned with the load axis of the actuator in the Bose ElectroForce® 3230 Test Instrument (Bose Corporation – ElectroForce Systems Group, USA). Great care was taken in order to assure full tissue clearance of the machine. Tendon thickness and width were measured with a digital micrometer for calculation of cross sectional area. Finally, ultimate tensile strength (UTS) was determined by failing the tendons under constant elongation at 20 mm/minute. The data were automatically stored and saved. Failure mode was determined by a visual and tactile inspection.

Statistics: Standard descriptive statistics were used to summarize the variables, i.e. means, standard deviation and percentage. $p \leq 0.05$ (two-tailed) was considered to be statistically significant. In some instances it was considered necessary to report the level of significance exact, i.e. when describing the results from correlation analyzes or discussing statistical tendencies. Due to the small sample size (n=6-10) and, thus, low power in the statistical analyses, it is recognized that only pronounced group differences could be statistically confirmed. Almost all data were found to be normally distributed and subsequently analyzed by the one way ANOVA test for group differences. This was followed by the Tukey´s HSD test for post hoc analyzes. The Student´s t-test was used where applicable. For the skewed variables, the Kruskal-Wallis test followed by the Mann-Whitney U test for pair-wise comparisons were used. The Pearson´s correlation coefficient was used to express the relationship between normally distributed variables, while the Kendall tau_b correlation coefficient was used for the skewed variables.

RESULTS

Weight: The general health status, including normal weight increase, of the rats was assessed over the course of the experiment (Fig. 2). The denervated group (*T*) demonstrated lower increases in weight as compared to the operated- (*C*) and the healthy, intact, controls (*C-0w*) ($p< 0.05$).

SP and CGRP levels at their production site, the dorsal root ganglia (DRG): The levels of SP/CGRP in the T-group were at 1 week 62% / 37% and at 4 weeks 64% / 36% lower ($p< 0.05$), respectively compared to the C-group, according to radioimmunoassay (RIA) (Fig. 3A-B). By 8 weeks the SP/CGRP concentrations had increased in the T-group such that no significant differences between the groups were detected.

SP and CGRP levels at the healing site: The peripheral presence of SP and CGRP was determined in the subcutaneous tissue close to the ruptured Achilles tendon, and assessed by semi-quantitative immunohistochemistry. The analysis showed that the neuropeptides SP and CGRP were depleted in small free nerve endings and perivascular nerve structures of the T-group compared to the C-group (Fig. 4A-F). The differences in neuropeptide occurrence between the groups decreased over time. The semi-quantification resulted in a significant difference ($p< 0.05$) at week 1, showing a lower CGRP occurrence in the T-group (0,01612) as compared to the C-group (0,29780). These data were strengthened by significant correlations between peripheral neuropeptide concentrations and their respective DRG levels. The peripheral neuronal presence of SP and CGRP at week 1 correlated with the corresponding levels in the DRG ($r=0.54$, $p=0.018$ and $r=0.71$, $p=0.001$).

Mechanical and Thermal Sensitivity: Hindpaw withdrawal latency (HWL) was assessed to confirm denervation and to relate nociception to central and peripheral levels of SP and CGRP. Both capsaicin treatment and surgery resulted in significant differences in HWL

between the groups and over time ($p< 0.001$). Prior to denervation, no significant differences between the groups in mechanical and thermal sensitivity were detected ($p>0.05$).

After completion of capsaicin-mediated denervation prior to tendon surgery, mechanical and thermal sensitivity declined in the T-group, as evidenced by significantly increased HWL, compared to controls ($p< 0.05$) (Fig. 5A-B). However, at 1 through 4 weeks post-tendon rupture there were no detectable differences in mechanical and thermal HWL between the T- and C-groups. At 6 and 8 weeks post rupture thermal sensitivity, and at 8 week mechanical sensitivity, again demonstrated a significantly decreased mechanical sensitivity in the T-group, as compared to the control groups ($p< 0.05$).

Biomechanical Properties: To evaluate the effects of capsaicin-induced SP depletion on the healing process, the development of tissue biomechanical properties was followed over the course of the experiment. The transverse area of the tendon at week 1 post rupture was significantly smaller in the T-group as compared to the C-group ($p< 0.05$) (Table 1). Subsequently during the healing process, the differences in transverse area decreased and became non-significant.

The ultimate tensile strength (UTS) at week 1 and 4 post tendon rupture exhibited equal values in the T- and the C-group (Table 1). Interestingly, at 8 weeks all samples in the T-group ruptured in the midsubstance of the tendon at a higher mean UTS than the C-group ($p< 0.01$), where all animals except one failed at the bone-tendinous junction. The differences in the distribution of the failure modes were significant (Table 2). Stress at failure, however, did not show any significant differences at any of the assessed time points between the groups (Table 1).

Due to the significant differences in failure mode between the groups, the high individual animal to animal variability, both in the normal levels of neuropeptides detected in the tissues and in the subsequent response to chemical denervation, correlation analyses were performed

on individual rats for weight, neuropeptide levels, pain sensitivity and biomechanical tissue properties.

Correlation of weight gain with biomechanical tissue properties: There were no significant correlations between weight increase and the biomechanical parameters measured, i.e. transverse area, UTS and stress at failure ($p>0.05$).

Correlation of SP and CGRP levels with biomechanical tissue properties: Only the concentration of SP demonstrated significant correlations with the biomechanical tissue properties. Thus, residual SP levels in the dorsal root ganglia of the T-group correlated as a group with the transverse area ($r=0.39$, $p=0.036$), UTS ($r=0.53$, $p=0.005$) and stress at failure ($r=0.43$, $p=0.023$), while SP in the DRG of the C-group did not show any correlations with the biomechanical tissue properties.

Correlation of pain sensitivity with peripheral levels of SP: Interestingly, high sensitivity to mechanical and thermal stimuli at weeks 2 and 4 was associated with increased peripheral occurrence of SP at week 4 in the T-group. In the C-group, there was also a correlation between mechanical sensitivity and the local occurrence of SP at week 4 (Table 3).

Correlation of pain sensitivity with the biomechanical tissue properties: Based on the above investigation of nociception correlating with peripheral SP levels, pain sensitivity was used as a surrogate of peripheral early SP levels and subsequently correlated with later biomechanical tissue properties (Table 4).

Interestingly, increased thermal sensitivity at week 2 exhibited a strong correlation with increased UTS and stress at failure at 4 weeks in the T-group. Moreover, thermal sensitivity at weeks 1 and 4 also had a tendency to correlate with UTS and stress at failure at week 4. In addition, in the T-group mechanical sensitivity at week 1 showed a trend to correlate with UTS and demonstrated a correlation with stress at failure at 4 weeks.

DISCUSSION

The results presented in this report demonstrate that higher residual substance P levels after capsaicin treatment are related to improved biomechanical tissue properties during healing after Achilles tendon rupture in this rat model.

Correlation of SP levels with biomechanical tissue properties

The most striking findings demonstrated that the SP levels in the dorsal root ganglia of the capsaicin treated animals correlated with the ultimate tensile strength (UTS) and stress at

failure. Importantly, the SP levels in the DRG correlated with peripheral SP levels. Notably, peripheral SP is known to stimulate proliferation of fibroblasts (33), and other studies have shown that exogenous local administration of SP promotes tendon and ligament repair (8, 19). Exogenous CGRP administration, however, did not enhance ligament repair (8). Thus, we speculate that peripheral SP levels, but not CGRP, promotes ultimate tensile strength and stress at failure during connective tissue healing.

Correlation of early peripheral SP levels with later biomechanical tissue properties

At the biomechanical level, the healing tissue properties in the late proliferative phase of healing at week 4 should, however, most likely depend on the synthesis of structural proteins from week 1 up to this time point, an outcome that could be related to the early neuropeptide levels. We established, consistent with other studies (26), a strong correlation between mechanical and thermal sensitivity and peripheral SP levels. The sensitivity to noxious stimuli was hence used as a surrogate for the early peripheral sensory neuropeptide levels. Thus, a strong correlation for thermal sensitivity at week 2, and a tendency to correlate for thermal sensitivity at week 1 and 4, with both UTS and stress at failure at week 4 was demonstrated. Such observations may reflect the findings that peripheral SP levels up to week 2 are critical for optimizing the subsequent biomechanical properties of the healing tissues at week 4. In fact, SP has been shown to exhibit a biphasic expression pattern during tendon healing, occurring during the inflammatory- and proliferative phase of healing respectively (25, 26).

Thus, the correlations of higher pain sensitivity during the inflammatory phase, i.e SP levels, and later improved biomechanical tissue properties may reflect that SP enhances vasopermeability, vasodilation and recruitment of leukocytes (34), and additionally may be involved in stimulating stem/progenitor cell activity. SP has, in fact, after corneal injury been demonstrated to stimulate stem/progenitor cell mobilization of stromal-like CD29 positive cells, which actively participate in wound healing (35).

While the correlations of higher pain sensitivity during the proliferative phase and the later improved biomechanical tissue properties may suggest that SP stimulates proliferation of fibroblasts (33), myofibroblasts, and possibly also stem/progenitor cells (35).

Effects of capsaicin treatment on sensory neuropeptides

The data presented clearly showed that chemical sensory denervation via subcutaneous administration of capsaicin was successful in reducing the concentrations of SP by ~60% and CGRP by ~40% at their production sites, i.e. the dorsal root ganglia (DRG), at 1 and 4 weeks post rupture, compared to the operated controls. Similarly, in the peripheral nervous system, capsaicin treatment significantly reduced the sensory neuropeptide levels at week 1, as also strengthened by the correlation with the neuropeptide levels in the DRG. Thus, the early reduction in SP levels throughout the proliferative phase of healing after capsaicin treatment demonstrates the validity of this model and provides a plausible explanation to the later observed biomechanical tissue effects.

The data from this study did not, however, demonstrate significantly reduced peripheral SP levels in the capsaicin treated group as compared to the control group at the 4 and 8 week timepoints. One explanation may be the successive restoration of SP concentration seen after capsaicin treatment. Other studies (36, 37), have shown that the reduction in SP after capsaicin treatment is maintained over time, which is consistent with our data regarding SP levels in the DRG. Moreover, the results from the mechanical and thermal stimulation tests also strengthen the concept of a significant prolonged functional denervation after capsaicin treatment.

While nerve transection admittedly would have provided a more uniform reduction in the levels of SP, the capsaicin treatment offers the advantage of no overt influence on motor function, i.e. no confounding effects due to changed mechanical stimulation of the healing

area (16, 38). The capsaicin model has been demonstrated to induce a ~50% reduction in the levels of SP and ~35% reduction of CGRP in peripheral sensory neurons under normal conditions (27), results which are in general agreement with our data. However, several studies demonstrate that even low concentrations of neuropeptides can exert tissue effects that may influence the repair process (39-41). This emphasizes the need to perform intra-individual correlations in the analyzes, analyses which clearly demonstrated that higher remaining substance P levels after capsaicin treatment were related to improved biomechanical tissue properties during healing.

Neuropeptide levels during healing and tissue homeostasis

Surprisingly, at the 8 week time point where the control group was normalized regarding the biomechanical properties and rupture mode, i.e failing at the tendon-bone insertion, the capsaicin treated group exhibited higher mean UTS, consistently failing in the tendon midsubstance. This observation implies that intact innervation, while improving the healing of the tendon midsubstance, may promote bone remodeling/resorption at the tendon-bone insertion (42-44). Since the control group failed in the bone-insertion site, it is not possible to comment on the UTS of the tendon mid-substance for this group. This striking and unanticipated observation of a homeostatic effect on the bony attachment has clear implications for the interpretation of prior studies of tendon healing where mechanical testing of the muscle-tendon-bone complex was done and the failure modes were not specified (43, 44).

Based on these findings, we hypothesize that in neurologically intact animals, tendon rupture induces a significant regional inflammation involving neurogenic factors. In addition to improving healing of the mid-substance rupture, these neurogenic factors also alter the bony insertion site, most likely by effecting bone homeostasis. In fact, SP is known to stimulate bone resorption (45-47), and we have previously observed that chronically inflamed tendons

exhibit an increased presence of nerves expressing SP and CGRP at the bone-tendinous junction (48).

Furthermore, an altered environment at the bone-tendon interface could also be induced by the fact that the tendon has lost its biomechanical loading once ruptured (de facto immobilized at the bone-tendon enthesis), which may lead to a de-repression of a subset of genes including inflammatory cytokines (49).

Denervation effects on mechanical loading

The significant increase in UTS at 8 weeks post rupture could also be due to a hypothetical increase in physical activity. Augmented mechanical stimuli of the healing area following the reduced nociception post-denervation could stimulate tendon healing and increase the tensile rupture load (50, 51). However, a previous study from our laboratory (31), demonstrated a very high level of physical activity almost immediately post rupture (authors´ observation, unpublished data) in animals with an intact nervous system, i.e. intact pain sensitivity. This would suggest that there are no overt differences between the pain sensitive and denervated group with regard to physical activity.

Conclusion

The present findings are consistent with the literature regarding an important role for neuropeptides in normal healing of a variety of diverse tissues (e.g. skin, cornea, bone, ligament) and indicate that the sparse innervation of the Achilles tendon appears to also play a similar critical role. These findings both corroborate the concept of a vital function for the sensory neuropeptides SP and CGRP during tendon tissue repair, as well as add new information to our understanding of their roles. Therefore, neural regulation of wound healing

appears to be a generalized activity in the healing process. Whether pharmacological or physical means of promoting neuronal pathways, i.e. optimizing the expression of sensory neuropeptides and their corresponding receptors, could be developed and employed after injury specifically in neuropathic patients has yet to be explored. Similarly, it is yet to be determined whether the healing process could be further enhanced by endogenous or exogenous stimulation via these mediators also in otherwise healthy patients. However, excessive influence of such mediators could contribute to pathologic healing (52, 53), so this concept must be investigated thoroughly to avoid any adverse sequelae.

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	BIOMECHANICAL PROPERTIES							
	C-0w	**T – 1w**		**C – 1w**	**T – 4w**	**C – 4w**	**T – 8w**	**C – 8w**
Area (mm^2)	1.8 ± 0.6	9.4 ± 1.7	*	12.7 ± 2.7	13.8 ± 1.3	15.3 ± 2.5	17.0 ± 1.9	16.7 ± 5.3
Ultimate tensile strenght (N)	41.2 ± 6.5	32.9 ± 9.7		32.5 ± 6.7	51.5 ± 17.1	61.0 ± 6.9	82.4 ± 19.9	* 51.9 ± 13.8
Stress (N/mm^2)	24.2 ± 6.1	3.6 ± 1.1		2.7 ± 0.8	3.7 ± 1.2	4.1 ± 0.7	4.9 ± 1.4	3.4 ± 1.6

Table 1.

Biomechanical tissue properties (area, ultimate tensile strength and stress) in the Achilles tendon of the denervated and surgically treated group (**T-**) and in the controls (**C-groups**), before rupture (**C-0w**) and after 1 (**T – 1w**; **C – 1w**), 4 (**T – 4w**; **C – 4w**) and 8 (**T – 8w**; **C – 8w**) weeks of healing. The values are expressed as mean ± SD. (n = 6). The asterix represents significant group differences (* = $p < 0.05$).

	FAILURE MODE							
	C-0w	**T – 1w**	**C – 1w**	**T – 4w**	**C – 4w**	**T – 8w**		**C – 8w**
Muscle tendinous junction	0	0	2	2	0	0		1
Mid substance	1	6	4	2	3	6	*	0
Bone tendinous junction	5	0	0	2	3	0		5
Tot (n)	6	6	6	6	6	6		6

Table 2.

Different tissue failure modes at ultimate tensile stress, in the muscle tendinous junction, the mid substance or in the bone tendinous junction in the Achilles tendon of the denervated and surgically treated group (**T-**) and in the controls (**C-groups**), before rupture (**C-0w**) and after 1 (**T – 1w**; **C – 1w**), 4 (**T – 4w**; **C – 4w**) and 8 (**T – 8w**; **C – 8w**) weeks of healing. (n = 6). The asterix represents significant group differences (* = $p < 0.05$).

MECHANICAL AND THERMAL SENSITIVITY – correlations with peripheral subcutaneous levels of SP	**SP levels**	
	T – w4	**C – w4**
Mechanical withdrawal latency **w 2**	r = −0.86, p = 0.003	r = -0,50, p = 0,143
Mechanical withdrawal latency **w 4**	r = −0.67, p = 0.048	r = −0.65, p = 0.041
Thermal withdrawal latency **w 2**	r = −0.68, p = 0.044	r = −0.56, p = 0.090
Thermal withdrawal latency **w 4**	r = −0.80, p = 0.010	r = 0.13, p = 0.72

Table 3.

Correlations between mechanical and thermal sensitivity (hind paw withdrawal latency) at 2 and 4 weeks and the peripheral local subcutaneous levels of substance P (SP) at 4 weeks of tendon healing, as assessed by immunohistochemistry, in the denervated (**T-group**) and in the controls (**C-group)**. (r = the correlation coefficient). (p = p-value).

MECHANICAL AND THERMAL SENSITIVITY – correlations with biomechanical tissue properties

	T– w4	
	Ultimate tensile strength (N)	**Stress (N/mm^2)**
Mechanical withdrawal latency **w 1**	r = −0.55, p = 0.091	r = −0.65, p = 0.046
Thermal withdrawal latency **w 1**	r = −0.59, p = 0.068	r = −0.49, p = 0.129
Thermal withdrawal latency **w 2**	r = −0.89, p = 0.006	r = −0.78, p = 0.015
Thermal withdrawal latency **w 4**	r = −0.62, p = 0.051	r = −0.52, p = 0.099

Table 4.

Correlations between mechanical and thermal sensitivity in the hind limb (hind paw withdrawal latency) at 1, 2 and 4 weeks and the biomechanical tissue properties (ultimate tensile strength and stress) at 4 weeks of Achilles tendon healing in the denervated (**T-**) and in the controls (**C-groups**). (r = the correlation coefficient). (p = p-value). A high sensitivity for noxious stimuli early on in the healing process correlated with a subsequent increase in the biomechanical tissue properties at a later time point.

FIGURE LEGENDS

Figure 1. Flow chart describing the structure of the experiment, i.e. the denervated and surgically treated group (**T-**) and the controls (**C-groups**) without and after surgery (**C-0w** and **C**, respectively). (✝ = animals lost during the experiments).

Figure 2. Weight development in the experimental groups, i.e. the denervated and surgically treated group (**T**, ♦, n = 8) and the controls without and after surgery (**C-0w**, ▲, n = 10 and **C**, ■, n = 10, respectively). The symbols represent the mean ± SD. The asterix represents significant differences between the **T**- (♦) and **C**-group (■) (* = $p < 0.05$).

Figure 3A-B. RIA tissue concentrations (pmol/g) of SP (**A**) and CGRP (**B**) in the dorsal root ganglion of denervated and surgically treated rats (**T**-) compared to the controls (**C-groups**), without rupture (**C-0w**, n = 10) and after 1 (**T – 1w**, n = 9; **C – 1w**, n = 10), 4 (**T – 4w**, n = 10; **C – 4w**, n = 10) and 8 (**T – 8w**, n = 8; **C – 8w**, n = 10) weeks of healing. The dots represent individual values and the horisontal bar represents the group mean. The missing values were samples with SP (**A**) and CGRP (**B**) concentrations below detection limit. The asterix represents significant group differences (* = $p < 0.05$).

Figure 4A-F. Immunofluorescence micrographs of sections of subcutaneous tissue from the right hind paw of denervated and surgically treated rats and the controls, before rupture (**C-0w, E-F**) and 1 week post tendon rupture (**T – 1w**, **A-B**; **C – 1w, C-D**) after incubation with antisera to SP (**A, C, E**) or CGRP (**B, D, F**). Arrows denotes varicosities and nerve terminals. A marked reduction of SP and CGRP immunoreactivity is seen in the denervated group (**A-B**) as compared to the controls (**C-D** and **E-F**).

lct = loose connective tissue, v = vessels. Bar = 50 µm.

Figure 5A-B. Time course of the development of sensitivity to mechanical (**A**) and thermal (**B**) stimulation in the experimental groups, i.e. the denervated and surgically treated group (**T**, ♦, n = 8) and the controls before and after surgery (**C-0w**, ▲, n = 10 and **C**, ■, n = 10, respectively), measured by hind paw withdrawal latency (HWL). The symbols represent the mean ± SD. The asterix represents significant differences between the denervated and surgically treated rats (**T,** ♦) and the control group (**C,** ■) (* = $p < 0.05$).