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Manual therapy as an effective treatment for fibrosis in a rat model of upper extremity overuse injury

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Abstract

Key clinical features of carpal tunnel syndrome and other types of cumulative trauma disorders of the hand and wrist include pain and functional disabilities. Mechanistic details remain under investigation but may involve tissue inflammation and/or fibrosis. We examined the effectiveness of modeled manual therapy (MMT) as a treatment for sensorimotor behavior declines and increased fibrogenic processes occurring in forearm tissues of rats performing an high repetition high force (HRHF) reaching and grasping task for 12 weeks. Young adult, female rats were examined: food restricted control rats (FRC, n=12); rats that were trained for 6 weeks before performing the HRHF task for 12 weeks with no treatment (HRHF-CON, n=11); and HRHF task rats received modeled manual therapy (HRHF-MMT, n=5) for 5 days/week for the duration of the 12-week of task. Rats receiving the MMT expressed fewer discomfort-related behaviors, and performed progressively better in the HRHF task. Grip strength, while decreased after training, improved following MMT. Fibrotic nerve and connective tissue changes (increased collagen and TGF-β1 deposition) present in 12-week HRHF-CON rats were significantly decreased in 12-week HRHF-MMT rats. These observations support the investigation of manual therapy as a preventative for repetitive motion disorders.

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Keywords

collagen; cumulative trauma disorder; fibrosis; massage; mobilization; repetitive strain injury; overuse injury; TGF-β1

Introduction

Work-related musculoskeletal disorders (WMSDs) are often termed work-related cumulative trauma disorders, overuse injuries and repetitive strain injuries, and include work-related carpal tunnel syndrome. The United States Occupational Safety and Health Administration estimates that WMSDs in the United States account for over 600,000 injuries and illnesses (OSHA 2014) and 34 percent of all lost workdays reported to the Bureau of Labor Statistics (BLS 2013). These disorders are estimated at $20 billion a year in direct costs, and up to 5 times more in indirect costs for MSD-related workers' compensation, in addition to the substantial toll on affected workers who develop significant difficulties in performing simple upper extremity tasks (OSHA 2014). Studies in humans with upper extremity WMSDs find evidence of inflammation, fibrosis and degeneration in tissues, changes thought to cause the concurrent sensorimotor dysfunctions (Ettema, Amadio et al. 2004, Rempel and Diao 2004, Carp, Barbe et al. 2007, Rechardt, Shiri et al. 2011, Riondino, La Farina et al. 2011, Chikenji, Gingery et al. 2014). There remains a call for effective, or ideally preventive, treatments for these often debilitating disorders (World Health Organization 2007, Bureau of Labor Statistics 2014, OSHA 2014).

The use of various manual therapy modalities for the treatment of carpal tunnel syndrome has been recently reviewed (Page, O'Connor et al. 2012), with the conclusion that there is only poor evidence supporting meaningful clinical efficacy of these modalities. However, two pilot reports on the effects of massage therapy on carpal tunnel syndrome report reduced symptoms and increased strength post-treatment (Moraska, Chandler et al. 2008, Elliott and Burkett 2013). Reviews of massage therapy (sports massage) for post-exertional muscle soreness are equivocal, yet overall clinical utility is supported (Moraska 2005). It is notable that most of the published literature reports results of short-term massage therapy treatment for repetitive motion disorders, which typically develop over weeks or even years. People with WMSDs tend to not use their affected limb, and disuse has been associated with increased fibrosis (Fink, Egl et al. 2007, Kaariainen and Kauhanen 2012). Although it follows that early treatment might prevent these changes, we could not identify any studies using manual therapies as a preventative for the development of carpal tunnel syndrome or other types of WMSDs.

Patients with chronic (>3 months) WMSDs show continued symptoms of pain and motor dysfunction, yet an absence of serum and tissue inflammatory markers, and instead, have increased tissue fibrosis and fibrogenic markers, such as transforming growth factor beta 1 (TGF-β1) (Freeland, Tucci et al. 2002, Ettema, Amadio et al. 2004, Hirata, Nagakura et al. 2004, Chikenji, Gingery et al. 2014). Although there is limited clinical evidence for a role of manual therapy in these processes, animal models have shown that passive movement allowed tendons to heal with less fibrosis (Gelberman, Menon et al. 1980, Gelberman, Woo

et al. 1982), and a model of active stretching showed findings of reduced subcutaneous collagen formation post-injury (Bouffard, Cutroneo et al. 2008).

We have developed a unique rodent model of operant repetitive reaching and grasping in which the performance of a reaching and handle-pulling task causes injury and inflammation, followed by nerve, muscle and connective tissue fibrosis, and then compressive nerve pathology with reduced nerve conduction velocity (Clark, Barr et al. 2003, Clark, Al Shatti et al. 2004, Elliott, Barr et al. 2009, Elliott, Barr et al. 2009, Elliott, Barr et al. 2010, Fedorczyk, Barr et al. 2010, Abdelmagid, Barr et al. 2012, Gao, Fisher et al. 2013, Jain, Barr-Gillespie et al. 2014, Fisher, Zhao et al. 2015). We observed exposure-dependent declines in sensorimotor function after short-term performance of these tasks (≤3 months), with a high repetition high force (HRHF) task inducing the greatest dysfunction (Fedorczyk, Barr et al. 2010, Barbe, Gallagher et al. 2013, Fisher, Zhao et al. 2015). We have shown that ibuprofen administered in weeks 5–6 of a 6-week HRHF task, or in weeks 5–12 of a 12-week HRHF task, attenuated inflammatory responses that drove tissue fibrosis (Abdelmagid, Barr et al. 2012, Jain, Barr-Gillespie et al. 2014). We have also shown that an anti-rat tumor necrosis factor alpha (TNFα) provided in weeks 5–6 of a 6-week HRHF task attenuated both tissue inflammatory and fibrogenic responses (Rani, Barbe et al. 2010, Abdelmagid, Barr et al. 2012). Unfortunately, long-term use of ibuprofen or anti-TNFα drugs can have negative side effects, including ibuprofen-induced gastrointestinal bleeding, renal toxicity, increased risk of myocardial infarction, and hypertension (Moore, Derry et al. 2007, Al-Saeed 2011), or serious infections with long-term blocking of TNFα function (Mohan, Scanga et al. 2001, Byun, Lee et al. 2015). Therefore, we are interested in exploring the effectiveness of non-pharmacological secondary prevention interventions.

Our goal here was to examine the effectiveness of manual therapy in preventing the fibrosis and reduced function that occur as a consequence of overuse. Our approach in this study was to emulate what would typically occur in the clinical setting, where the person develops symptoms and seeks care. This qualifies as a “secondary prevention” approach (Amick, Tullar et al. 2006).

Methods

Subjects

The Temple University Institutional Animal Care and Use Committee approved these experiments in compliance with NIH guidelines for the care and use of laboratory animals. Female Sprague-Dawley rats (3 mo. of age at onset of experiments) were used because human females have a higher incidence of work-related musculoskeletal disorders (Gerr, Marcus et al. 2002, Ratzlaff, Gillies et al. 2007, Srilatha, Bhat et al. 2011, Cote 2012), and to allow comparison to our past studies on same-aged female rats using this model, such as those examining the effectiveness of anti-inflammatory drugs in reducing HRHF-task induced inflammation (Rani, Barbe et al. 2010, Abdelmagid, Barr et al. 2012, Jain, Barr-Gillespie et al. 2014).

All rats were housed in AAALAC-accredited animal facility in separate cages with a 12-hour light:dark cycle with free access to water and environmental enrichment in their home

cages (chew toys and tunnels). All rats were handled extensively for 1 week prior to onset of experiments, and then 5 days/week thereafter. All rats were food-restricted to body weights of 5% less than age-matched normal controls (used only for weight comparison) in order to motivate interest in food reward pellets. Rats were weighed weekly and allowed to gain weight across the course of the experiment, as shown previously, since they were young adults. In addition to 45 mg food pellet rewards (a 1:1 mix of purified grain and banana flavored pellets; Bio-Serv, NJ, USA), all rats received Purina rat chow daily (i.e. same food reward and chow rations for all groups).

A power analysis was performed using Statview using prior tissue TGF-β1 and grip strength data (Abdelmagid, Barr et al. 2012, Fisher, Zhao et al. 2015). We chose the most conservative sample size needed to detect differences with an alpha level of 0.05 and 80% power. The power analysis estimated that for ANOVAs of tissue and motor behavioral findings that a sample size of at least n=5/group was needed, per type of tissue analyses (histological versus biochemical). Since not all rats destined for the HRHF task learn the task completely, two additional rats each were added the FRC group and HRHF group. Based on this, we first randomly divided rats into three groups: 1) age-and weight-matched food restricted control rats (FRC, n=7); 2) age-matched rats that were trained for 6 weeks before performing the high-repetition, high-force task (HRHF) task for 12 weeks with no treatment (HRHF-CON, n=6); and 3) age-matched rats that were trained for 6 weeks before performing the HRHF task for 12 weeks that would receive modeled manual therapy (HRHF-MMT, n=6). After training, one rat in the HRHF groups did not train adequately, and were removed from the study, leaving n=6 in the HRHF-CON group and n=5 in the HRHF-MMT group. Later, histological findings of only low inflammation in the 12-week HRHF-CON rats required confirmation; so 5 additional rats per group were added to the FRC and HRHF-CON groups for biochemical analyses. By study end, data from 28 rats were used: FRC rats, n=12; HRHF-CON rats, n=11; HRHF-MMT, n=5.

Behavioral Apparatuses, Training, and Task Regimen

Sixteen custom-designed behavioral apparatuses were used for these experiments, as previously described and depicted (Barbe, Gallagher et al. 2013). Briefly, animals reached through a shoulder height portal and isometrically pulled a force handle attached to a force transducer with a load cell (Futek Advanced Sensor Technology, Irvine, CA) located outside the chamber wall. The load cell was interfaced with custom Force-Lever software (version 1.03.02, Med Associates, St. Albans, VT). Auditory and light indicators cued the reaching rate (defined below). If reach and force criteria (defined below) were met within a 5 second cueing period, a 45 mg food pellet was dispensed into a trough.

All 17 HRHF rats underwent an initial training period for 6 weeks in which they learned the task, as previously described in detail (Barbe, Gallagher et al. 2013). Briefly, rats were initially food-restricted for 7 days to no more than 10% less than their naïve weight, and the weight of age-matched normal control rats with free access to food (used for weight comparisons only, and not included in this study), to initiate interest in food reward pellets. After that week, they were given extra rat chow to gain weight quickly back to only 5% less than the normal control rats. Rats were weighed weekly, maintained at ± 5% less than the

normal controls; all were allowed to gain weight during the study, as reported previously (Jain, Barr-Gillespie et al. 2014). The 17 rats underwent operant training to learn the reaching and handle-pulling task during a 5-week period of 10 min/day, 5 days/wk, in which they ramped upwards from naive towards the HRHF task level. All but two of the rats undergoing training reached the HRHF level (described below) during their last week of training. The 16 rats that learned to reach at the HRHF level went on to the next stage of the experiment (HRHF-CON, n=11; HRHF-MMT, n=5).

After the training period, a point equal to week 0 of the HRHF task, all 15 trained rats began the HRHF task regimen for 2 hrs/day, 3 days/wk for up to 12 weeks. The task was divided into 4, 0.5-hr sessions separated by 1.5 hrs in order to avoid satiation. HRHF rats were cued to reach at a rate of 4 reaches/min and to extend their forearm into a portal, grasp a handle, and then exert a target isometric pull for at least 50 milliseconds (ms) at a force effort of 40% ± 5% group maximum (40% maximum pulling load = 77 cN) (See Supplemental Fig 1). These criteria had to be met within a 5 second window initiated every 15 seconds, and the handle held with the correct force for 50 ms. If these criteria were met, rats received a food reward deposited into a food trough, and this was considered a successful reach. Rats were allowed to use their preferred limb to reach (the “reach” limb), and data from this limb only is reported.

Modeled Manual Therapy

Because people often seek treatment when they first develop symptoms, we initiated the MMT after training to the high force level, when rats begin to develop symptoms consistent with WMSDs (Rani, Barbe et al. 2010, Abdelmagid, Barr et al. 2012, Barbe, Gallagher et al. 2013, Fisher, Zhao et al. 2015). One group of HRHF rats (HRHF-MMT; n=5) underwent MMT beginning in week 0 after training, and then 5 days/week for the duration of the 12 weeks of HRHF task performance. The FRC and HRHF-CON rats underwent light handling only instead of the MMT treatment for the 12-week duration.

The MMT treatment protocol was developed by an experienced manual therapist (GMB) and taught to the technician (MH) who provided the training, data collection, and care in these and previous studies. The technician had no previous experience with massage therapy delivery. She observed the protocol over a few hours, and then studied video recordings of the initial training sessions across several days; her performance of the MMT was then videotaped for supervision and adjustment as needed by the experienced manual therapist. The protocol, designed to emulate what a massage therapist or other manual therapist might perform, consisted of scaled-down versions of many common modalities. These included (in order) gentle mobilization, skin rolling, and deep strokes (aka “muscle stripping” or “myofascial release”) to the forearm flexor compartment; joint mobilization of the wrist; and stretching combined with long superficial strokes of the entire upper limb (see video in Supplemental Information).

Unsedated rats were placed in the lap of the seated operator, and held gently until comfortable (1–2 minutes). The scapula and shoulder were then stabilized with one hand while the other hand delivered the treatment in 5 phases.

1. The thumb, index finger, and middle finger gently compressed the flexor muscles, and mobilized them laterally over the radius and ulna (10 cycles).
2. The skin over the forearm was pinched together and rolled between the thumb and index finger (5 rolls), including all loose skin from the wrist to the elbow.
3. While one hand stabilized the wrist, the index finger of the hand stabilizing the shoulder was used to push the skin distally over the flexor muscles, and then was pressed into the flexor tendons and muscles and pulled proximally. Enough force was used to be able to feel the deeper structures but not enough to cause the rat to withdraw its paw. This provided deep spreading pressure to the muscles and tendons in all but the most proximal part of the flexor compartment. This was repeated 10 times.
4. The distal forearm was stabilized with one thumb and index finger, while the other thumb and index finger stabilized and lightly tractioned the paw. The wrist was mobilized 5 cycles posterior to anterior, and 5 cycles in axial rotation, while maintaining the traction of the paw.
5. While the rat was stabilized at the shoulder, the treating thumb and fingers grasped the proximal upper limb and gently tractioned (a stroking traction) while sliding across the fur, rolling back and forth when reaching the forearm and continuing this to the rat's fingers until they slid out of the treating hand.

Treatment was performed on both arms, and took less than 10 minutes per session depending on the cooperation of the individual rat.

Spontaneous changes in behaviors occurring during or after treatment

Although the rats seemed very comfortable with the treatments, we looked for and recorded on incidence any behaviors indicative of discomfort. These potential behaviors included: momentary freezing, prolonged freezing, strong escape behaviors, limb withdrawal during touching or mobilization, increased defecation or urination, increased biting or acts of aggression, and increased vocalization. Rats were then returned to their home cage, and for 1 hour after each treatment session, home-cage behaviors that could be related to pain or discomfort were recorded on incidence, including: excessive movement around the home cage, increased grooming, limb guarding, paw licking, or limping. Similar home-cage behaviors were also observed for in untreated FRC and HRHF-CON rats, for 1 hour per day, 5 days/week for 12 weeks.

Spontaneous changes in behaviors occurring during task performance

Trained observers tracked changes in spontaneous behaviors occurring during each period of HRHF task performance. Bilateral pulling of the lever bar was recorded upon occurrence, as was a switch in forearm used to pull the lever bar from the typically used "preferred" reach limb. On occasion, a rat would twist its forelimb into a supinated position when pulling on the lever bar rather than using a typical pronated position; this was recorded as a "supinated pull." Fumbling, guarding, or pulling with one digit rather than the typical four-digit grasp were recorded upon occurrence, as was refusal to participate (typically seen as sitting in the corner of the chamber, and thus termed "sits in corner"). Data from the last day of each task

week are reported for HRHF-CON and HRHF-MMT groups (FRC rats did not perform the task).

Force lever data were also recorded continuously during each task session for later calculation of the percentage of successful reaches via an automated script (MatLab; Mathworks, Natick, MA). A reach was defined as a force deflection that exceeded 2.5% of the baseline (or zero) force until the next sample in which the force fell below 2.5% of baseline. The number of successful reaches/week is reported for the HRHF-CON and HRHF-MMT groups (FRC rats did not perform the task).

Reflexive Grip Strength

Reflexive grip strength of the forelimbs was measured in 15 animals (FRC, n=10; HRHF-CON, n=10; HRHF-MMT, n=5) bilaterally, at baseline (the naive time point), after training (the 0 week time point of the HRHF task), and every 2 weeks thereafter, using a rat grip strength-recording unit (Stoelting, Wood Dale, IL), as described previously (Kietrys, Barr et al. 2011). These assays were performed by an examiner naive to group assignment. The test was repeated 5 times/limb/trial, and the maximum grip strength per trial reported in centinewtons (cN).

Serum biomarker assays

Serum was collected from 15 rats in the study for assay of TGF-β1 levels (FRC, n=10; HRHF-CON, n=10; HRHF-MMT, n=5). At 90 minutes after the last task session, rats were anesthetized with 5% isoflurane in oxygen and then euthanized by exsanguination. Blood was withdrawn from the heart via cardiac puncture and centrifuged immediately at 1000 x g for 20 min at 4°C. The supernatant (serum) was collected and stored at −80°C until analyzed using a commercially available ELISA kit for TGF-β1 (KAC1688, Invitrogen Corporation, Camarillo, CA). Results from the serum ELISA were normalized to ml of serum (pg TGF-β1/ml serum).

Histological and Immunohistochemical Analyses

Forelimb tissues were collected for histological analysis from FRC (n=7), HRHF-CON (n=6), and HRHF-MMT (n=5) rats were collected. Following euthanasia and after serum collection, animals were perfused transcardially with 4% buffered paraformaldehyde (pH 7.4). Tissues were collected and postfixed *en bloc* by immersion for two days. Forepaws were then separated from the forelimbs at the level of the wrist.

Forepaws were removed at the level of the wrist using a small Dremel bone saw. The forepaw tissues were processed *en bloc*, with skin, lumbricals and bone intact, and embedded in paraffin, sectioned into 5 μm thick cross-sections, mounted onto charged slides (Super Frost Plus, Thermo Fisher Scientific, Pittsburgh, PA), and allowed to dry overnight before storage at room temperature. After deparaffinization in xylene and rehydration, sections on slides were stained with Masson's Trichrome, dehydrated, coverslipped with DPX mounting medium for bright field microscopy, and examined for fibrous collagen (stained blue). Adjacent sections were treated with 3% H_2O_2 in methanol to block for endogenous peroxidase for 30 min, washed in phosphate buffered saline (PBS), then

permeabilized with 0.05% pepsin in 0.01N HCL, washed again in PBS, prior to blocking with 4% dried milk (Carnation) in PBS for 20 min at room temperature. Sections were then immunostained in batched sets by the same individual for TGF-β1 using a mouse monoclonal anti-TGF-β1 at a 1:500 dilution in PBS with 4% milk (MAB240, R&D Systems, Minneapolis, MN). Slides were incubated overnight at room temperature in a humid chamber. On the 2nd day, after washing, sections were incubated with goat anti-mouse secondary IgG antibody conjugated to HRP (Jackson Immunoresearch Laboratories, West Grove, PA; diluted 1:100 in PBS, incubated 2 hours at room temperature before washing in PBS) and visualized using DAB (Fast DAB, Sigma, St. Louis, MO). These sections were dehydrated, and then coverslipped with DPX mounting medium for brightfield microscopy. A third set of adjacent sections was stained with hematoxylin and eosin.

After removal of the forepaw at the level of the wrist, the fixed forearm flexor digitorum muscle and tendon mass were removed from the forearm bones using a scalpel. A proximal region near the elbow was removed with a scalpel for cross-sectioning, while the remaining flexor digitorum muscles were separated *en bloc* as a flexor mass for longitudinal sectioning. These tissues were cryoprotected in 30% sucrose in PBS before cryosectioning into 15 μm cross sectional or longitudinal slices. Sections were then placed onto charged slides (Super Frost Plus, Thermo Fisher Scientific) and allowed to dry overnight before storage at −80°C. These cryosectioned tissues were immunostained in batched sets by the same individual for collagen type I and TGF-β1 using sequential double-labeling methods [mouse monoclonal anti-Collagen type I at 1:500 dilution (C2456, Sigma) and mouse monoclonal anti-TGF-β1 at 1:500 dilution (MAB240, R&D Systems)]. Briefly, after an antigen retrieval step of 0.5% pepsin for 15 minutes at room temperature, sections were washed in phosphate buffered saline (PBS), incubated for 30 minutes in 10% goat serum in PBS, before incubating with anti-Collagen type I at the listed dilution for overnight at room temperature. Sections were washed in PBS before incubation in a goat anti-mouse secondary IgG antibody, that had been preabsorbed to reduce non-specific cross-reactivity with rat antigens, and conjugated to a red fluorescent cyanine dye (AffiniPure $F(ab')_2$ fragment, CY3, 550 nm excitation; Jackson ImmunoResearch Laboratories) at a dilution of 1:100 for 2 hours at room temperature in the dark. Sections were washed in PBS and reblocked in 10% goat serum in PBS for 20 min, before incubating overnight at room temperature with anti-TGF-β1 at the listed dilution. Sections were washed in PBS before incubation in a goat anti-mouse secondary IgG antibody conjugated to a green fluorescent cyanine dye (DYLIGHT 488; Jackson ImmunoResearch Laboratories) at a dilution of 1:100 for 2 hours at room temperature in the dark. The fluorescent tag-labeled sections were washed with PBS, incubated with DAPI (1:1000 dilution in PBS) for 15 minutes at room temperature, washed again, then coverslipped with 80% glycerol in PBS for epifluorescence microscopy. Adjacent sections were stained with hematoxylin and eosin.

Preabsorption control staining was also performed to demonstrate if the antibodies bound specifically to the antigen of interest using TGF-β1 human recombinant protein (GF111, Millipore, Billerica, Massachusetts) or Collagen type 1 purified rat protein (C7661, Sigma, St. Louis, MO). No labeling was observed in the tissues for any pre-absorbed antibody, as previously published and depicted (Fisher, Zhao et al. 2015). We also performed no-primary

antibody controls in which serum was substituted for the primary antibody, followed by secondary antibodies; no labeling was observed, as previously published and depicted (Fisher, Zhao et al. 2015).

Quantification of staining and immunostaining was performed in batched sets by one person (MFB), blinded to group assignment, using a microscope (Nikon E600) interfaced with a digital camera (Retiga 4000R QImaging Firewire Camera, Surry, BC Canada) and an image analysis system (BioQuant Osteo II, Bioquant Image Analysis Corporation, Nashville, TN). First, in the forepaw and median nerve at the level of the wrist, the proportion of each field or region of median nerve, respectively, displaying the blue stain after Masson's Trichrome staining (indicative of fibrous collagen) was quantified. The microscope's light intensity (bright field), camera gain and exposure were standardized and remained constant. The blue color was designated as a separate threshold in the RGB component of the image BioQuant software. This threshold was saved in a subprogram, and then applied to all images for consistent auto-measurement of the blue stain. The Videocount Area Array option of the software was also utilized (defined as the number of pixels in a field that met a user-defined color threshold of staining). The number of thresholded blue pixels in each field were then counted and analyzed as a percentage of the total number of pixels in that field, using similar methods as previously described for liver fibrosis (del Pilar Alatorre-Carranza, Miranda-Diaz et al. 2009). For the glabrous region of dermis of the mid forepaw, 3 to 4 fields were counted per animal (in cross-sectional tissue slices) using a 20X objective, and the % area of blue staining (fibrous collagen) was averaged for each animal, before comparing results across groups. For the median nerve at the level of the wrist, 2–3 fields were counted per animal (in longitudinal sectioned tissue slices) using a 10X objective, and the % area with blue staining averaged for each animal, before comparing results across groups.

Next, the percent area of immunoreactivity for TGF-β1 in the paraffin embedded forepaws was performed in batched sets in the glabrous dermis and adjacent underlying loose connective tissue, and around the lumbricals, in 5 animals/group. The % area of immunoreactivity for TGF-β1 and collagen 1 in the cryosections were also quantified in batched sets in the proximal flexor digitorum muscle region that had been cross-sectioned, and in longitudinal sections of flexor digitorum tendons (near the wrist). The microscope's epifluorescence light intensity, camera gain and exposure were standardized, and remained constant for each batch of acquired images. Again, a thresholded color was chosen (green or red), saved into a subprogram for consistent auto-measurement of green or red pixels in the field, versus total number of pixels in each field. Three to 5 fields were counted per region, per animal, using a 20X objective, and the % area with staining was averaged for each animal, before comparing results across groups.

Tissue Biochemical Assays

Following euthanasia and after serum collection, flexor digitorum muscles from 5 FRC rats and 5 HRHF-CON rats were collected from the distal forearm region of the preferred reach limb. Muscles were homogenized, as previously described (Barbe, Gallagher et al. 2013), and assayed by customized multiplex bead-based analysis kits (LXSARM, R&D Systems)

for IL-1α, IL-6, IL-1β, and TNFα. ELISA assay data (pg of cytokine protein) were normalized to μg total protein, determined using a bicinchoninic acid protein assay kit.

Statistics

Behavior data across weeks was assayed using SAS (SAS Institute Inc, Cary, NC). Specifically, a Poisson regression with generalized estimating equation was used to estimate incidence rate, odds ratios, and 95% confidence intervals (CI) for spontaneous behaviors occurring in reach versus nonreach limbs of HRHF-MMT rats during MMT. A linear mixed-effects model was used to estimate and compare the incidence rate of altered movement strategies across weeks in HRHF-CON versus HRHF-MMT rats. Logistic regression with generalized estimating equation was used to compare differences in individual altered movement strategies in HRHF-CON versus HRHF-MMT rats. A linear mixed-effects model was also used to estimate and compare the change over time (slopes) in the number of successful reaches, and the maximum grip strength, between groups (FRC, HRHF-CON and HRHF-MMT). One-way ANOVAs were used to compare results of histochemical and immunohistochemical quantification, except for quantification of fibrosis in the median nerve, where a two-tailed t-test was used, using GraphPad PRISM v.6.02, GraphPad Software, Inc., La Jolla, CA. Bonferroni post-hoc corrections were used when multiple pair-wise tests were performed on the same data set to reduce the chances of obtaining false-positive results (Type I errors). In accordance with this method, an adjustment was made to *p* values by the analysis program used by dividing the critical p value ($\alpha = 0.05$) by the number of comparisons made, thus increasing the stringency of the analysis. Adjusted p values of < 0.05 were considered significant for all comparisons. Group means plus standard error of the mean (SEM) or standard error (SE) are reported.

Results

Daily MMT led to a small increase in withdrawal of the preferred reach limb during MMT, and no observation of spontaneous behaviors suggestive of discomfort

For the 5 HRHF-MMT rats, Poisson regression analysis showed a small increase in spontaneous behaviors suggestive of discomfort was observed during MMT of the reach limbs, compared to the non-reach limbs [incidence rate ± SE for the reach limb = 0.65 ± 0.26; nonreach limb = 0.92 ± 0.23; incidence rate ratio (95% CI) = 0.7 (0.5, 0.99); p=0.047]. When these behaviors were examined individually, the HRHF-MMT rats showed no increased urination, defecation, or vocalization behaviors during MMT of either limb (Fig. 1), and no biting or acts of aggression (data not shown). There was a very small but equal incidence of strong escape behaviors during MMT of either limb in weeks 7 and 9 (Fig. 1), although these increases did not reach significance in post hoc tests.

Our monitoring of spontaneous signs of discomfort continued after return of rats to the home cage (excessive movement around the home cage, increased grooming, limb guarding, paw licking or limping). None were observed. These observations indicate that the treatment was well tolerated.

Spontaneous behaviors suggestive of discomfort occurring during HRHF task were less with MMT

Since we have previously observed increased spontaneous behaviors suggestive of discomfort during performance of this HRHF grasping and pulling task (Fisher, Zhao et al. 2015), these same behaviors were monitored here. A linear mixed-effects model was used to show that the incidence rate of altered movement strategies that may indicate discomfort or reduced function were lower across weeks of task performance in HRHF-MMT rats, compared to HRHF-CON rats (Fig. 2; HRHF-MMT overall incidence rate mean ± SEM = 1.38 ± 0.22; HRHF-CON = 1.97 ± 0.17; difference = −0.59 ± 0.26; p=0.027). Logistic regression analyses of individual subgroups of altered movement behaviors showed a decreased incidence rate of limb switching in HRHF-MMT rats, compared to HRHF-CON rats [Odds ratio (95% CI) = 0.026 (0.0066, 0.0999); p<0.001]. Also, guarding/avoidance behaviors did not occur in HRHF-MMT rats after Week 3, and pulling with only 1–2 fingers (rather than all 4 fingers) was not observed in HRHF-MMT rats after Week 7 (Fig. 2); low incidence of each were observed in HRHF-CON rats. Fumbling was observed only in 1 HRHF-CON rat in Week 4. These observations suggest that the treatment was beneficial to task performance.

Modeled manual therapy led to enhanced performance and reduced motor declines typically induced by the HRHF task

While the number of successful reaches per week increased slightly over time in the HRHF-CON rats, performance of the rats receiving MMT was significantly enhanced (Fig. 3A). A linear mixed-effects model was used to show that the slope of change in successful reaches across weeks of task performance in HRHF-MMT rats was 10.6 ± 2.95 (slope ± SE); in contrast, the slope in HRHF-CON rats was 1.08 ± 2.95 (difference = 9.5 ± 4.18; p=0.025). Post-hoc tests comparing the number of successful reaches per week showed that these effects were driven by the higher incidence of successful reaches by HRHF-MMT rats at testing weeks 4–12 (Fig. 3A). These data support a substantial increase in voluntary task performance due to the MMT treatments.

Consistent with our previous studies, grip strength in the preferred reach limbs of each HRHF group decreased during training and continued to decline with time in HRHF-CON rats, compared to FRC rats (Fig. 3B). A linear mixed-effects model was used to show that the slope of change in grip strength in HRHF-CON rats was −11 ± 1.66 cN/week (slope ± SE), while the slope in FRC rats −2.5 ± 1.57 cN/week (difference = −8.5 ± 2.29, p=0.0003). During this same task period, the grip strength of HRHF-MMT rats improved towards levels seen in FRC rats (Fig. 3B; HRHF-MMT slopes ± SE = −3.5 ± 2.24 cN/week, difference between HRHF-MMT and FRC rats = −1.02 ± 2.74, p=0.71) and away from levels seen in HRHF-CON (Fig. 1B; difference between HRHF-MMT and HRHF-CON slopes ± SEM = 7.5 ± 2.79 cN/week, p=0.008). Post-hoc tests showed that these effects were driven by the decreased grip strength of HRHF-CON rats at all points after training compared to age-matched FRC rats, but decreases in HRHF-MMT rats, compared to FRC rats, only post training (week 0) and through task week 2 (Fig. 3B). These data support an increase in reflexive forearm/forepaw motor strength due to the MMT treatments.

Maximum pulling force did not induce inflammation in HRHF rats by 12 weeks of task performance

H&E staining did not show increases in inflammatory cells in any tissues examined in the HRHF-CON and HRHF-MMT rats at the time of tissue collection (12 weeks after onset of HRHF task performance), compared qualitatively to FRC rats (n=5/group; data not shown). To further confirm a low inflammatory response in tissues, additional FRC and HRHF-CON animals (n=5/group) were examined using ELISA and then one-way ANOVAs for the presence of pro-inflammatory cytokines in flexor digitorum muscles (IL-1α, IL-6, IL-1β, and TNFα). No significant increases were observed in HRHF-CON rats, compared to FRC rats (data not shown).

Modeled manual therapy prevented HRHF-induced increased forepaw collagen deposition around the median nerves and lumbrical muscles

Consistent with our previous results showing increased collagen deposition in forepaw and forelimb tissues of HRHF rats (Clark, Al Shatti et al. 2004, Fedorczyk, Barr et al. 2010, Abdelmagid, Barr et al. 2012, Gao, Fisher et al. 2013, Jain, Barr-Gillespie et al. 2014, Fisher, Zhao et al. 2015), HRHF-CON rats had increased fibrous collagen deposition in the forepaw connective tissues surrounding branches of the median nerves, lumbrical muscles and their individual myofibers in HRHF-CON rats (Fig. 4A,B), compared to HRHF-MMT rats (Fig. 4C,D). As shown in Figures 4F and G, there was also increased collagen deposition around and within the median nerve at the level of the wrist (i.e., intraneurally between individual axons). These differences were confirmed by quantification of the blue staining in this latter region using a two tailed t-test ($p=0.007$; Fig 4E). Similar increases in fibrous collagen deposition were observed in their glabrous forepaw dermal extracellular matrix of HRHF-CON rats, compared to both FRC and HRHF-MMT rats (Fig. 5A,C-F). These differences were confirmed by quantification of the blue staining in this region (n=5/group; ANOVA, $F_{2, 12} = 25.33$, $p < 0.001$; Fig. 5B).

Modeled manual therapy attenuated HRHF task-induced increases in tissue TGF-β1 and collagen type 1

We observed increased TGF-β1 immunoreactivity in the dermis and underlying loose connective tissue of the glabrous dermal region of the forepaw in HRHF-CON rats, compared to FRC and HRHF-MMT rats (Fig. 6A,C, E). These differences were confirmed by quantification of the percent area with TGF-β1 immunostaining in this region (n=5/group; ANOVA, $F_{2, 12} = 12.45$, $p = 0.001$ ($p<0.05$ each posthoc test, Fig. 6G). The TGF-β1 immunoreactivity was localized within and around enlarged dermal fibroblast-like cells in HRHF-CON rats (Fig. 6C; representative cells are indicated by arrows). In the nearby lumbrical muscles, we observed low level increases TGF-β1 immunoreactivity in small fibroblast-like cells located on the perimeter of myofibers in HRHF-CON rats, compared to FRC and HRHF-MMT rats (Fig. 6B, D, F; representative cells are indicated by arrows). These differences were confirmed by quantification of the % area with TGF-β1 immunostaining in the lumbrical muscles (n=5/group; ANOVA, $F_{2, 12} = 19.59$, $p = 0.0002$ ($p<0.05$ each post hoc test; Fig. 6H). The latter increases were over 3 fold less than that seen in the connective tissue of the dermis.

Using cross-sections and longitudinal sections of mid-belly flexor digitorum muscles for immunohistochemistry, we examined TGF-β1 and collagen type I immunoreactivity. We found low levels in each of the FRC rats (Fig. 7A–C), higher levels in HRHF-CON rats (Fig. 7D–F), yet lower levels in HRHF-MMT rats, qualitatively, compared to HRHF-CON rats (Fig. 7G–I). The collagen type I immunostaining was localized to fibroblast-like cells and extracellular matrix immediately surrounding the myofibers, particularly in HRHF-CON rat tissues (Fig. 7D,F), while TGF-β1 immunostaining was localized just to the fibroblast-like cells in the perimeter of myofibers. These findings were quantified in cross-section slices of mid-belly flexor digitorum muscles and showed that the % area with TGF-β1 immunoreactivity in the mid-flexor digitorum muscle region was increased in HRHF-CON rats (n=6), compared to FRC (n=7) and HRHF-MMT rats (n=5; ANOVA, $F_{2, 15} = 9.05$, $p = 0.002$; Fig. 8A). The percent area with collagen type 1 immunoreactivity in this same region of mid-flexor digitorum muscle was also quantified and shown an increase in HRHF-CON rats, compared to FRC and HRHF-MMT rats (ANOVA, $F_{2, 15} = 10.92$, $p = 0.001$; Fig. 8B).

We also assayed serum for TGF-β1 levels using ELISA and observed increased serum TGF-β1 levels in HRHF-CON rats (n=5), compared to HRHF-MMT rats and compared to FRC rats (n=10 each; ANOVA, $F_{2, 22} = 10.22$, $p = 0.0007$; Fig. 8C). Note though, that this difference was driven mainly by an increase in serum TGF-β1 in two HRHF-CON rats.

Data from Figures 4–8 support that the MMT treatments prevented increases in collagen deposition and TGF-β1 production in forearm and forelimb tissues.

Discussion

In our well-characterized rat model of work-related musculoskeletal disorders (Fedorczyk, Barr et al. 2010, Abdelmagid, Barr et al. 2012, Barbe, Gallagher et al. 2013, Fisher, Zhao et al. 2015), we have shown that a multimodal intervention designed to emulate manual therapy, applied in the early stages of symptom development, prevents functional declines, improves task performance, and prevents behavioral changes indicative of discomfort during task performance. We have also shown that MMT attenuated the increased fibrosis and tissue levels of TGF-β1 in and around nerves and muscles that characterize this model and that correlate with the sensorimotor declines (Jain, Barr-Gillespie et al. 2014, Fisher, Zhao et al. 2015). Although our findings are on a very small number of treated rats, the results were beneficially unidirectional, supporting a robust response to MMT.

Two pilot studies report that 6 weeks of massage therapy for the treatment of carpal tunnel syndrome reduced symptoms and increased strength (Moraska, Chandler et al. 2008, Elliott and Burkett 2013). There is also a case report of two patients with lateral epicondylopathy in which pain symptoms were reduced after 4–6 weeks of manual therapy (Papa 2012). Reviews of massage therapy (sports massage) for post-exertional muscle soreness are equivocal, but overall clinical utility is supported for reduction of delayed onset muscle soreness, stiffness, and fatigue (Hilbert, Sforzo et al. 2003, Wilson and Best 2005, Zainuddin, Newton et al. 2005, Ogai, Yamane et al. 2008, Han, Kim et al. 2014, Urakawa, Takamoto et al. 2015). Most published literature reports results of short-term treatment of MSDs (a single bout at < 1 week after onset of muscle soreness). We could not identify any

studies using manual therapies as a preventative for the development of chronic upper extremity RMDs, although one animal study shows that active stretching of the back (10 min/day for 12 days) improved gait and lowered mechanical hypersensitivity in a mouse model of induced low back pain (Corey, Vizzard et al. 2012). Findings from several animal models show that passive movement allows tendons to heal with less fibrosis (Gelberman, Menon et al. 1980, Gelberman, Woo et al. 1982), and that stretching after microsurgical injury reduces subcutaneous collagen formation (Bouffard, Cutroneo et al. 2008). Studies using instrumented massage of skeletal muscle (single bout or up to 4 days) after nerve stimulation-induced maximum eccentric exercise showed increased muscle viscoelastic properties, and reduced muscle stiffness and muscle fiber damage (Haas, Best et al. 2012, Haas, Butterfield et al. 2013, Haas, Butterfield et al. 2013, Crawford, Haas et al. 2014). Findings from these studies combined with our current data support further investigations of MMT for WMSDs.

In the untreated HRHF-CON rats, we observed increases of a key fibrogenic protein, TGF-β1, and collagen type 1 in extracellular matrix around and within the median nerve and muscles, and in the dermal connective tissues of the forepaw. TGF-β1-immunostained cells in muscles were most likely fibroblasts. Other groups have shown that fibrogenic proteins increase in fibroblasts under conditions of tissue overload or injury (Best, Shehadeh et al. 2001, Kjaer 2004, Nakama, King et al. 2006, Heinemeier, Olesen et al. 2007, Smith, Stauber et al. 2007). In a model of subcutaneous injury-induced fibrosis, mechanical stretching of 20–30% for 10 min/day for 7 days, reduced subcutaneous collagen formation, which was associated with attenuated TGF-β1 production following similar tissue stretching *ex-vivo* (Bouffard, Cutroneo et al. 2008).

Our findings of functional declines concomitant with fibrotic responses in the HRHF-CON rats match findings from patients with chronic (>3 months) WMSDs in which fibrotic responses are increased (Freeland, Tucci et al. 2002, Ettema, Amadio et al. 2004, Hirata, Nagakura et al. 2004). It has been postulated that fibrosis in and around muscles and nerves may distort dynamic biomechanical properties and increase tissue strain due to adherence to adjacent structures, reducing dynamic tissue function (Driscoll and Blyum 2011). Fibrosis in the connective tissue “container” surrounding nerves has been linked to chronic nerve inflammation (Bove, Weissner et al. 2009) and compression (O’Brien, Mackinnon et al. 1987), which are known to increase pain behaviors (as a consequence of compressive nerve irritation), evoke ectopic mechanical sensitivity and ongoing activity in nociceptor axons (Bove, Ransil et al. 2003, Bove 2009), and decrease grip strength (due to reduced nerve conduction). Similarly, we observed increased collagen deposition around myofibers, in and around nerve processes, and within the epimyseum, each supportive of an increased fibrotic “container” due to overuse. Our present findings support the hypothesis that fibrotic tissue changes are contributing to the observed sensorimotor behavioral declines.

Other treatments evaluated in this rat model include ibuprofen (oral, 40 mg/kg body weight, provided in either weeks 5–6 or 5–12 of a 6-or 12-week HRHF task regimen) (Kietrys, Barr et al. 2011, Abdelmagid, Barr et al. 2012, Jain, Barr-Gillespie et al. 2014), an anti-rat TNFα blocking antibody (i.p.,15 mg/kg body weight, provided in weeks 4.5–6 of a 6-week HRHF task regimen) (Rani, Barbe et al. 2010, Abdelmagid, Barr et al. 2012). The 2-and 8-week

ibuprofen treatment reduced TGF-β1 production, and reduced aberrant collagen production and deposition in connective tissues surrounding nerve and muscles (Abdelmagid, Barr et al. 2012, Jain, Barr-Gillespie et al. 2014). Ibuprofen treatment improved the rats' duration of voluntary task performance, voluntary pulling force, and percent of successful reaches through week 9 (Kietrys, Barr et al. 2011). However, the improvement in % success was lost in week 12, and reflexive grip strength declines were only partially attenuated in ibuprofen-treated rats (Kietrys, Barr et al. 2011, Abdelmagid, Barr et al. 2012). This is perhaps because of continued presence of low-grade degenerative changes in the flexor digitorum myofibers in the HRHF+IBU rats (Jain, Barr-Gillespie et al. 2014), or a consequence of ibuprofen's reported negative effects on muscle metabolism and no contribution to improved muscle function (Tokmakidis, Kokkinidis et al. 2003, Soltow, Betters et al. 2006, Machida and Takemasa 2010). Anti-rat TNFα treatment also reduced TGF-β1 and collagen type 1 production (Abdelmagid, Barr et al. 2012), but only partially attenuated sensorimotor declines. This is perhaps because the drug was administered after development of pain behaviors and TNFα is an inducer of spinal cord sensitization thought to be involved in chronic pain, but not its maintenance, making anti-TNFα treatment after development of chronic pain behaviors unsuccessful (Sasaki, Kikuchi et al. 2007, Zhang, Zhang et al. 2013). As stated in the introduction, long-term use of ibuprofen can induce gastrointestinal bleeding, renal toxicity, increased risk of myocardial infarction, and hypertension (Moore, Derry et al. 2007, Al-Saeed 2011), and serious infections can occur with long-term anti-TNFα treatment (Mohan, Scanga et al. 2001, Byun, Lee et al. 2015).

Treatment effects of manual therapy are often ascribed to non-specific effects or the meaning response (Moerman and Jonas 2002). It is possible that the effects we observed were due to such nonspecific effects, since we did not have an active sham group (FRC controls were handled only daily), but we feel that this is highly unlikely. Each group of rats in these experiments was handled extensively by the researcher performing the treatments, usually daily, for 7 weeks prior to initiating the MMT; handling continued 5 days/week until completion of the experiments. Conditioned place preference experiments have documented that rats are capable of remembering many types of experiences (Navratilova, Xie et al. 2013), and so were likely to have been able to anticipate their treatments, which occurred daily. Since there were no behaviors associated with the treatments that were indicative of fear, it seems very unlikely that these animals were subjected to any adverse psychological stress. However, this model may prove useful in future studies to evaluate effects, if any, of this type of treatment on the HPA axis.

Study Limitations

Our study is on a small number of subjects, and the experiment needs to be reproduced prior to making assumptions on how such an approach would translate to human health care. It should be noted that the methods used have virtually no negative side effects, so there is little potential for harm in replicating this preventive approach with humans. While the methods were designed to be scaled-down versions of typical manual therapy treatments, it is unknown how the forces would be scaled up to human treatments. This may be a critical issue since in the field (based on GMB's personal experiences), treatments described as very similar can be applied with highly variable forces. Measurements of practitioner forces

during such soft tissue therapy are just beginning to appear (Best, Crawford et al. 2014, Wang, Zeng et al. 2014). Similar measurements from our approach could then be compared.

Since the protocol was designed to be multimodal, treating all the structures in the forearm, we do not know which of the sub-treatments led to the changes, although it makes sense that the skin rolling helped prevent the fibrotic changes within the interface between the skin and muscle, which occurred in the HRHF-CON rats. We also cannot tell which of the many changes are directly related to the symptoms. It seems likely that the fibrosis affecting the median nerve and its branches could be key to the pathophysiology in this model by induction of a persistent nerve inflammation and compressive pathology. Because we do not know which modality was effective, and because clinical manual therapy research is plagued by lack of consensus regarding sham treatments (Chaibi, Saltyte Benth et al. 2015), we chose to have a control group that was held only, and not otherwise actively treated. Further research is planned to address these challenging questions, as well as possible changes in angiogenesis and vascular function reported to occur with massage therapy (Andrzejewski, Kassolik et al. 2014, Franklin, Ali et al. 2014, Andrzejewski, Kassolik et al. 2015).

Conclusions

Our results show that a multimodal intervention designed to emulate manual therapy, applied in the early stages of symptom development, prevents functional declines, improves task performance, and prevents behavioral changes indicative of discomfort during task performance. The results also show that MMT attenuated the increased fibrosis that characterizes this model and that is associated with the sensorimotor declines (Fisher, Zhao et al. 2015), as well as the tissue and serum levels of TGF-β1, a key fibrogenic factor. Thus, manual therapy may be an effective preventative for MSDs by decreasing tissue fibrosis. Such research promises to shed light on the pathophysiology of WMSDs but more importantly, has the potential to translate into effective prophylactic treatments for people suffering from these common and often debilitating disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

MMT	Modeled manual therapy
cN	centiNewton
ECM	extracellular matrix

FRC	food-restricted control
HRHF	high repetition high force
ms	milliseconds
MSDs	musculoskeletal disorders
TGF-β1	transforming growth factor beta
TNFα	tumor necrosis factor alpha
WMSDs	work-related musculoskeletal disorders

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Highlights

1. Provision of modeled manual therapy for 5 days/week for the duration of a 12-week task paradigm reduced discomfort-related behaviors, improved grip strength, and enhanced task performance in rats performing a high repetition high force task.
2. The modeled manual therapy, performed 5 day/wk for 12 weeks, attenuated task-induced increases in collagen and TGF-β1 deposition in nerve and connective tissues of the forearm.
3. These observations support the investigation of manual therapy as a preventative for hand and wrist work-related musculoskeletal disorders, including median nerve fibrosis.

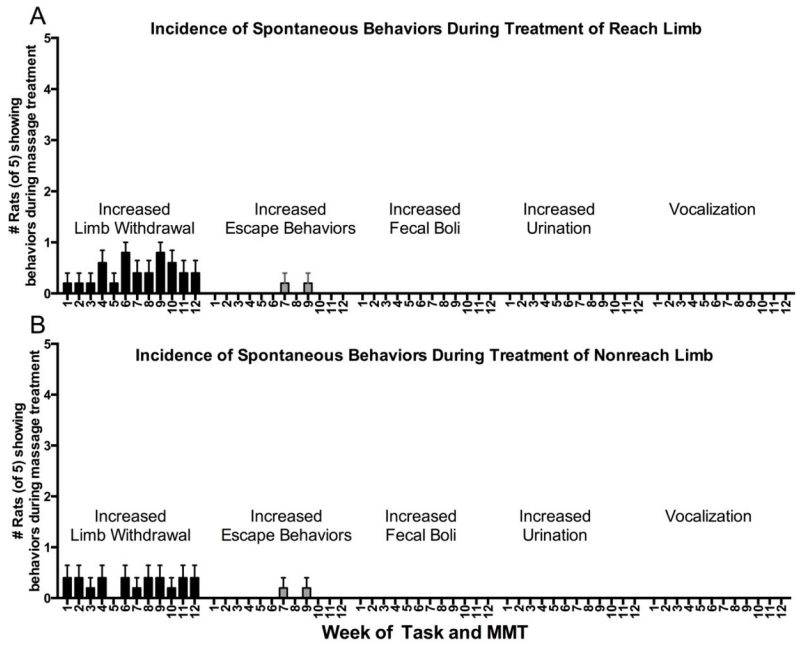

Figure 1.

Spontaneous behaviors observed during or after MMT in (A) reach and (B) non-reach limbs. A small increase in the incidence of increased limb withdrawal was observed during MMT of the reach limb, although there were no significant post hoc findings for the individual weeks. No increased incidences of increased escape to touch, defecation, urination, or vocalization behaviors were observed during MMT.

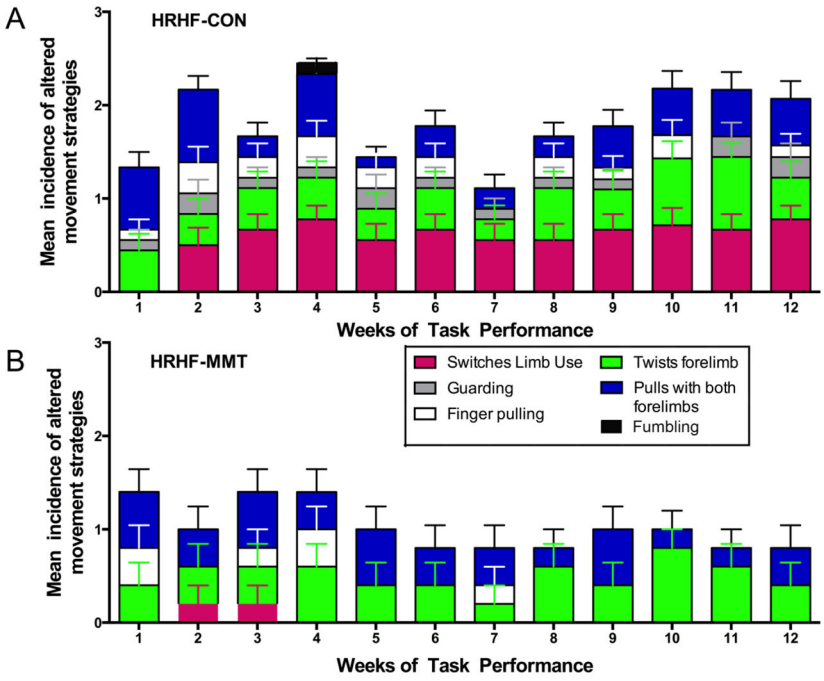

Figure 2.

Incidence of altered movement strategies (mean ± SEM) occurring during task performance in untreated HRHF task rats (HRHF-CON), and in HRHF task rats undergoing modeled manual therapy (MMT) for 12 weeks while continuing to perform the HRHF task (HRHF-MMT). (A) HRHF-CON rats (n=10). (B) HRHF-MMT rats (n=5). Significant decreases in the incidence of altered movement strategies ($p<0.027$), specifically, limb switching ($p<0.001$) were observed in HRHF-MMT rats, compared to HRHF-CON rats. Non-significant differences in the incidence of guarding, finger pulling, twisting of the forelimb, pulling with both forelimbs, and fumbling were observed in HRHF-MMT rats, compared to HRHF-CON rats.

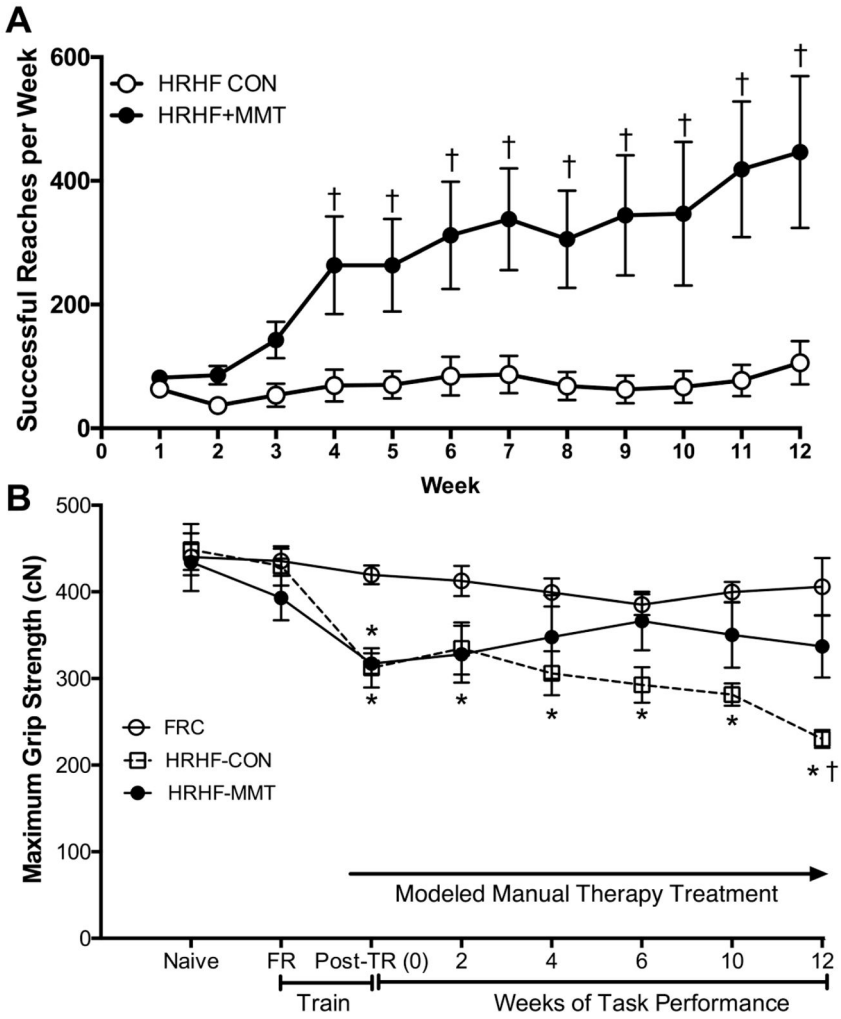

Figure 3.

Voluntary and reflexive motor behavioral declines with continued task performance. (A) The number of successful reaches per week in HRHF-CON rats (n=10), and HRHF-MMT (n=5) rats over 12 testing weeks. Successful reaches increased significantly over time in HRHF-MMT rats, compared to HRHF-CON rats ($p<0.05$). (B) Maximum reflexive grip strength (cN), tested using a rat grip strength meter, in food-restricted control rats (FRC, n=10), HRHF-CON (n=10) and HRHF-MMT (n=5) rats at the naïve time point, after food restriction, after training (week 0), and across the 12 weeks of the experiment. Grip strength declined significantly over time in HRHF-CON, compared to HRHF-MMT ($p<0.001$) or FRC ($p<0.001$). Post-hoc analyses at each week *: $p<0.05$, compared to age-matched FRC rats; †: $p<0.05$, compared to HRHF-MMT rats.

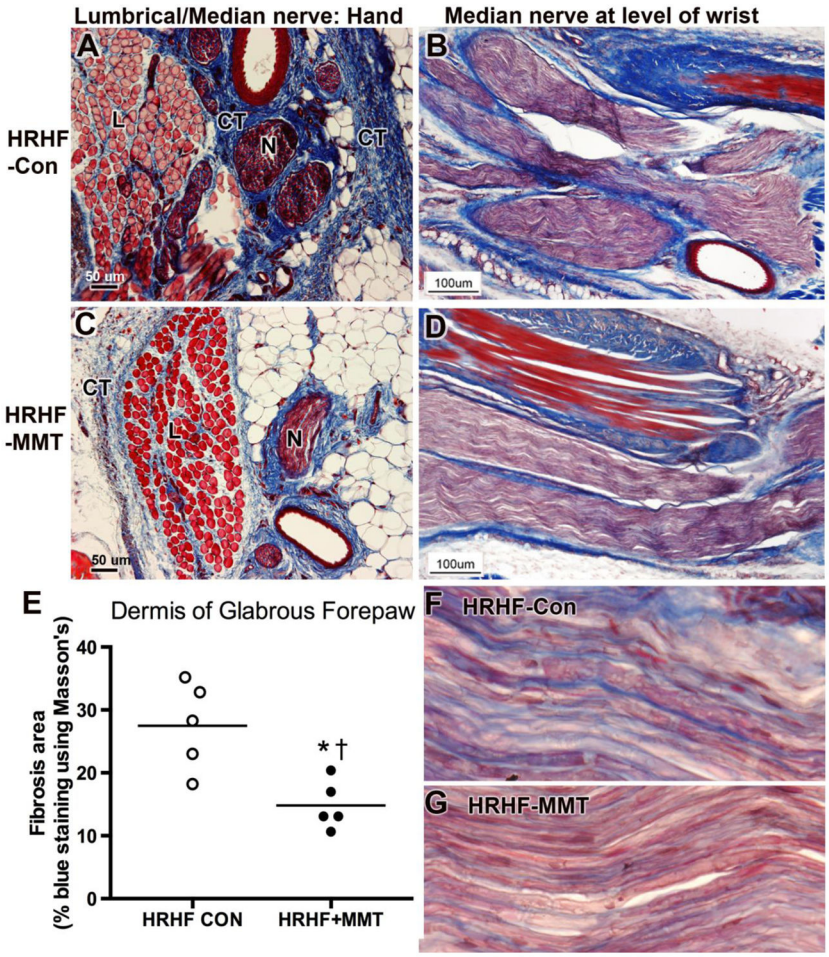

Figure 4.

Branches of the median nerve and lumbricals in cross-sections and longitudinal sections, after Masson's trichrome staining. Collagen fibrils are stained blue in HRHF-CON rats (A, B) and HRHF-MMT rats (C,D). (E) Quantification of percent blue-stained fibrous collagen around longitudinally sectioned median nerves, at the level of the wrist. Individual rat means, and overall means per group, are shown. †: $p<0.05$, compared to HRHF-MMT rats. (F and G) Higher power images of longitudinal sections of the median nerve at the level of the wrist, showing increased collagen deposition (blue staining) between individual myelinated axons of HRHF-CON rats, than in HRHF-MMT rats. Symbols: CT = connective tissue, L = lumbricals, N = median nerve branches. Scale bar = 50 μm in A and C, and 100 μm in B and D.

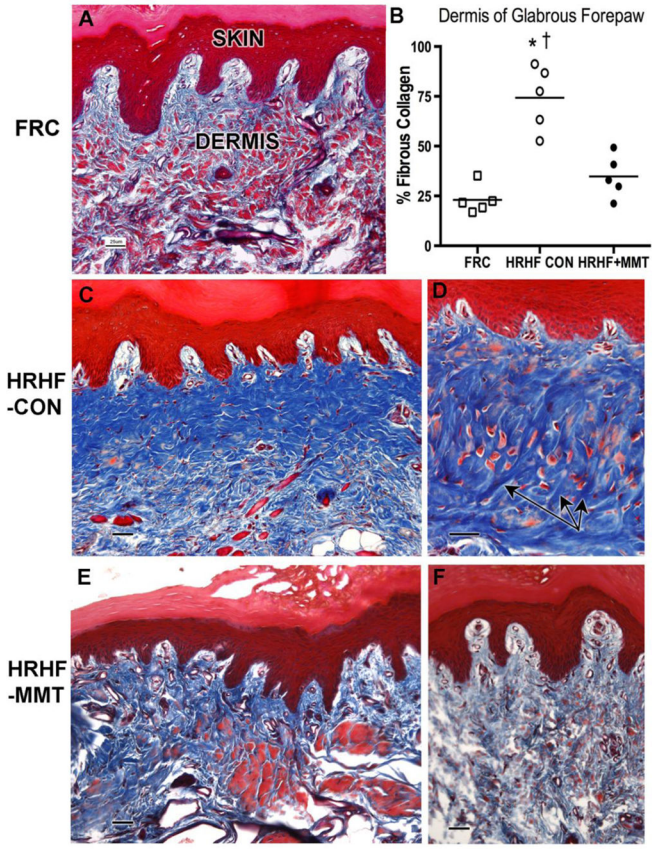

Figure 5.

Forepaw glabrous skin and underlying dermis after Masson's trichrome staining. Collagen fibrils are stained blue, in FRC, HRHF-CON, and HRHF-MMT rats. (A) Forepaw dermis and skin of FRC rat. (B) Quantification of percent blue-stained fibrous collagen in this region. Individual rat means, and overall means per group, are shown. Symbols = *: $p<0.05$, compared to FRC rats; †: $p<0.05$, compared to HRHF-MMT rats. (C–D) Images of forepaw dermis and skin from two different HRHF-CON rats. Arrow in D indicates "cords" of collagen fibrils in dermis. (E–F) Images of forepaw dermis and skin from two different HRHF-MMT rats. Scale bars on left hand sides of images = 25 μm.

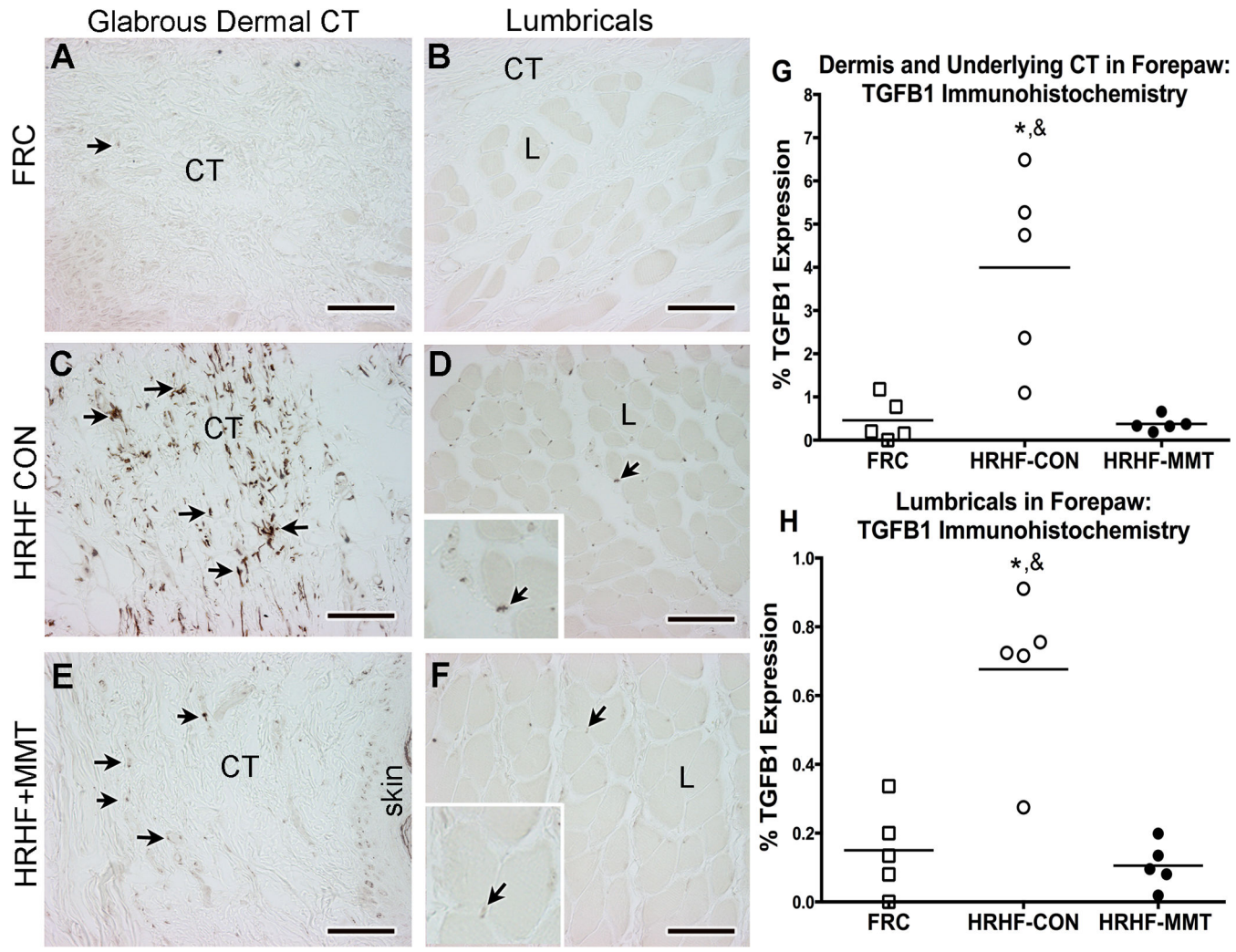

Figure 6.

TGF-β1 immunostaining in glabrous dermal connective tissue (CT) and lumbricals of forepaw, distal to the wrist joint. Cross sectional slices and images of TGF-β1 HRP-DAB (representative brown staining indicated by arrows) are shown. (A,B) Images from FRC rats. (C,D) Images from HRHF-CON rats. (E,F) Images from HRHF-MMT rats. CT = connective tissue, L = lumbricals. (G,H) Quantification of percent TGF-β immunostaining in the glabrous dermal region of the forepaw and lumbrical muscles. *: $p<0.05$, compared to FRC rats; †: $p<0.05$, compared to HRHF-MMT rats. Scale bar = 25 μm.

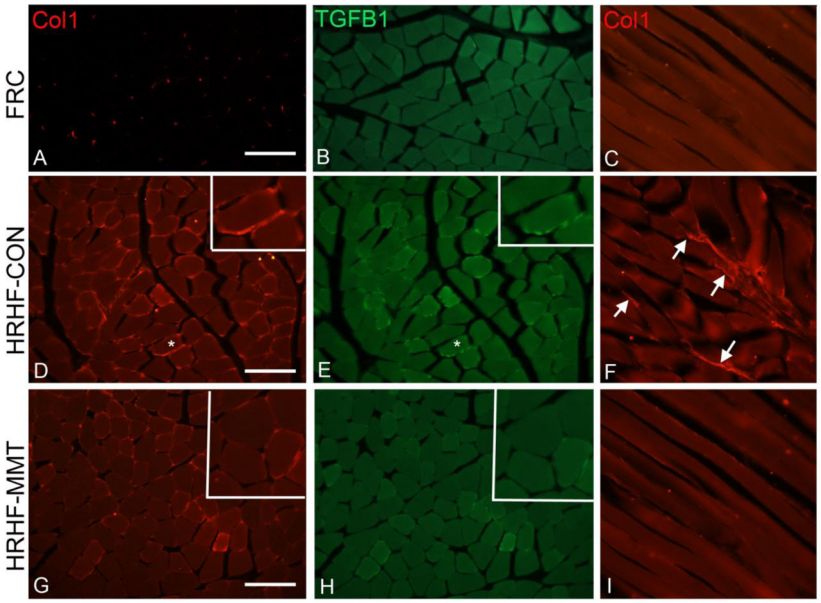

Figure 7.

Collagen type I immunostaining (red) and TGF-β1 immunostaining (green) in flexor digitorum muscles of FRC, HRHF-CON and HRHF-MMT rats. Cross sectional slices shown in panels A,B,D,E,G and H; longitudinal sections shown in panels C,F and I. (A–C) Images from FRC rats showing low immunoreactivity for each analyte. A and B are from the same field. (D–F) Images from HRHF-CON rats showing high immunoreactivity of each analyte on perimeter of individual myofibers. D and E are from the same field. * indicates cross-section of a myofiber that is enlarged in the inset. Arrows in panel F indicate immunopositive collage type 1 staining between myofibers. (H–I) Images from HRHF-MMT rats showing lower immunoreactivity of each analyte, than observed in HRHF-CON rats, on perimeter of individual myofibers. G and I are from same field. Inset shows an enlarged myofiber. Scale bars shows in A, D and G = 25 μm, and apply to other panels as well. *: $p<0.05$, compared to FRC rats; †: $p<0.05$, compared to HRHF-MMT rats.

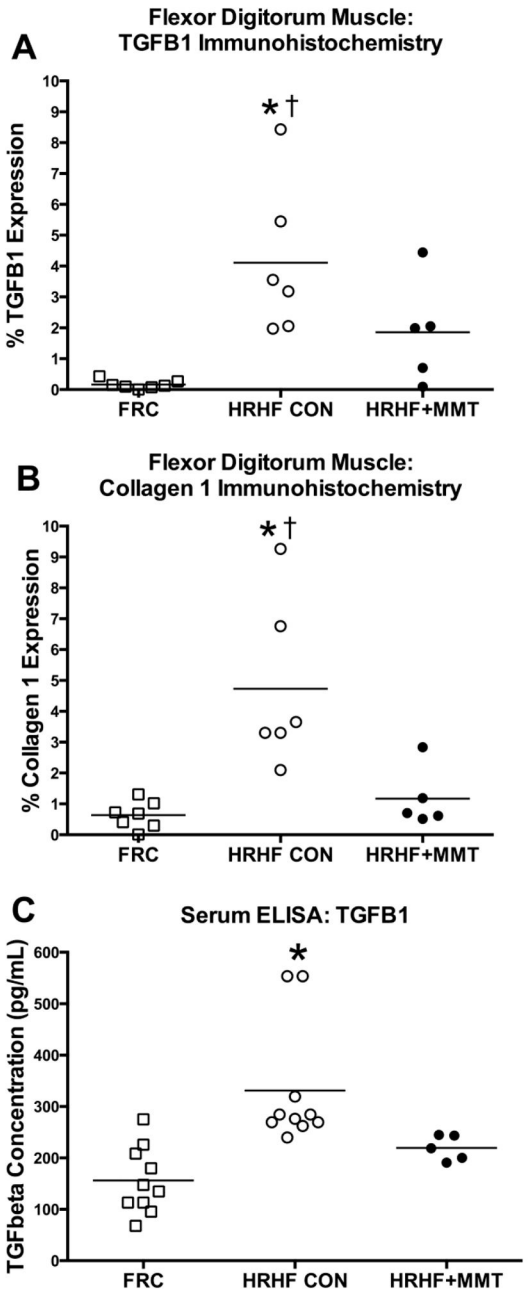

Figure 8.

Quantification of TGF-β1 in serum using ELISA, and quantification of TGF-β1 and collagen type 1 immunostaining in flexor digitorum muscles of FRC, HRHF-CON and HRHF-MMT rats. Individual rat means, and overall means per group, are shown in graphs. (A) Quantification of TGF-β1 immunostaining in cross sections of mid-belly flexor digitorum muscles. (B) Quantification of collagen type 1 immunostaining in cross sections of mid-belly flexor digitorum muscles. (A) Serum levels of TGF-β1. *: $p<0.05$, compared to FRC rats; †: $p<0.05$, compared to HRHF-MMT rats.