INCREASED SERUM TNF-ALPHA AND MATRIX METALLOPROTEINASE-2 ARE ASSOCIATED WITH GRIP STRENGTH DECLINES AND TISSUE DEGENERATION IN A RAT MODEL OF OVERUSE

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We have shown that continued performance of repetitive tasks induces grip strength declines despite resolution of systemic inflammation. We hypothesize this is due to underlying tissue degeneration. Here, we assessed long term performance (18 weeks) of a high-repetition, low-force (HRLF) task in a rat model of reaching and grasping. We observed reduced grip strength immediately after training, and persistent grip strength declines in reach limbs of HRLF rats. Several inflammatory cytokines increased in serum of 6- and 12-week HRLF rats, e.g. tumor necrosis factor alpha (TNF-alpha). TNF- alpha was also increased in reach limb muscles and tendons at similar time points. A serum analyte of collagen degradation (matrix metalloproteinase-2, MMP2) was increased in serum of 18-week HRLF rats. MMP2 and several other MMPs, as well as two fibrogenic proteins (CTGF and TGFB1), were increased in 18-week HRLF tendons, which also showed histological signs of pathology. Thus, motor declines were associated earlier with tissue inflammation but later with tendon degenerative changes. Assaying for TNF- alpha and MMP2 provided important insights into the stages of inflammation and degradation in this model.

Introduction:

According to the Bureau of Labor Statistics report entitled Nonfatal Occupational Injuries and Illnesses Requiring Days Away from Work for 2010, work related musculoskeletal Disorders (WMSDs) account for 29% of lost workday injuries and illnesses in the US (BLS, 2010). Studies in humans with long-term chronic overuse syndromes find evidence of inflammation, fibrosis and degeneration of musculoskeletal tissues. However, the factors underlying these pathophysiological responses are still under investigation. This impedes progress towards their prevention.

Several studies have detected biomarkers of inflammation in serum of patients with newly diagnosed WMSDs, including TNF-α and members of the interleukin 1 (IL-1) family (Carp et al, 2007; Rechardt et al, 2011). Although not yet assessed in patients with WMSDs, serum MMP2 has been shown to be a sensitive biomarker of extracellular matrix turnover and collagen degradation in animal models (Garner et al, 2011). It would be of interest to identify biomarkers of inflammation and degeneration of musculotendinous tissues, so that treatment of WMSDs can be targeted appropriately.

In a rat model of WMSDs, performance of a high repetition negligible force (HRNF) food-retrieval task for 8 weeks induced increased inflammatory cytokines in muscles and tendons that persisted across the 8 weeks of task performance (Barbe et al, 2008). The increase in tissue inflammatory cytokines was matched by a persistent increase of similar cytokines in serum. In a recent study, we observed that performance of a high repetition low force (HRLF) lever-pulling task for 12 weeks induced a serum cytokine response in week 6 that appeared to be resolved by week 12 in young adult rats (Xin et al, 2011). However, several questions remain, including whether the serum cytokine responses observed in earlier weeks are associated with tissue inflammatory responses, and if the serum inflammatory cytokine increased again with continued work. Since we have also observed persistent motor deficits with this HRLF task (Xin et al, 2011; Kietrys et al, 2012), we hypothesize that there is either a persistent low-grade inflammatory response or degradative changes in the involved muscles or tendons that are contributing to the motor deficits.

Our goal here was to determine if declines in forearm grip strength occurring with long-term overuse activity at moderate task levels are associated with inflammatory or degenerative responses in forearm muscle and tendons. We also sought to identify serum biomarkers indicative of tissue inflammatory, degradative tissue processes. We assessed forearm grip strength, serum for biomarkers of inflammation or tissue degeneration, and flexor digitorum muscles and tendons for indicators of inflammation, degradation and fibrosis in rats performing a voluntary HRLF lever-pulling task for 18 weeks.

Methods:

Subjects. The Temple University Institutional Animal Care and Use Committee approved all experiments in compliance with NIH guidelines for the care and use of laboratory animals. Seventy young adult, female Sprague-Dawley rats (3 mo of age at onset) were used. Rats were divided into one of 3 groups: One group consisted of age-matched normal controls (NC, n=15). A 2nd group consisted of trained only rats that underwent the initial training, and then were either euthanized immediately after the training period (TR0: n=10), or that then rested for 12 weeks (n=11, TR12) or, 18 weeks (n=6, TR18) before euthanasia. These trained animals did not proceed to the task regimen and served as age-matched trained controls. The 3rd group consisted of high repetition low force task (HRLF) rats that trained for 3-4 wks to learn the task, and then performed the HRLF task for 6 (n=6), 12 (n=10), or 18 weeks (n=12) before euthanasia.

Training and the Repetitive Task. The trained only and HRLF rats underwent a 4 week training period of 10 min/day, 5 days/week, to learn the task. Subsets of these rats were randomly chosen to become the HRLF task rats. The HRLF rats reached and pulled a lever at a rate of 4 reaches/min at 15

± 5% of maximum voluntary force, for 2 hrs/day in 30 min sessions, for 3 days/wk, for up to 18 wks. Details of this training and task are as described previously (Xin et al, 2011). Rats were allowed to use their preferred limb to reach (the ''reach'' limb), and their contralateral limb as support against the operant chamber wall while pulling (the "support" limb).

Motor function assay: Reflexive grip strength was measured in all animals bilaterally at baseline, after training, and every 3 weeks thereafter, using a rat grip strength recording unit (Stoelting, Wood Dale, IL). The test was repeated 3-5 times/limb/trial, and maximum grip strength per trial was reported and used for statistical comparisons.

Serum assays. Following euthanasia (Nembutal, 120 mg/kg body weight), 18 hours after completion of the final task session, blood was collected from all rats by cardiac puncture using a 23-gauge needle and centrifuged immediately at 1000 g for 20 min at 4^{o}C. Serum was collected, flash-frozen, and stored at -80^{o}C until analyzed. Serum was assessed for: C-reactive protein (CRP); Interleukin (IL)-1α and β, IL-12, macrophage inflammatory protein (MIP) 2 and 3; matrix metalloproteinase 2 (MMP2), and tumor necrosis factor-alpha (TNF-α), using ELISA (Aushon Biosystems). All samples were analyzed in duplicate, and presented as pg/ml serum.

Tissue biochemical assays. Following euthanasia and after serum collection, one half of the animals/group were used for biochemical assays, using previously described methods (Barbe et al, 2008)]. Homogenates were assayed for: TNF-α, MIP2, TGFB1, and MMP2 using ELISA, following manufacturers' instructions (Aushon Biosystems and Biosource). Each sample was run in duplicate and data normalized to pg/microgram total protein. Western blot analysis of CTGF was performed as described previously (Abdelmagid et al, 2012).

Immunohistochemical Analyses. The remaining animals/group were used for histological analysis. Following euthanasia and after serum collection, animals were perfused transcardially with 4% buffered paraformaldehyde. Forearm musculotendinous tissues were collected and sectioned longitudinally, as described previously (Barbe et al, 2003). Sections were immunolabeled for CTGF (connective tissue growth factor), TGFB1 (transforming growth factor beta 1), MMP1, MMP2, MMP8, and MMP13 proteins using immunohistochemical methods (Abdelmagid et al, 2012).

Assessment of Tendon Histopathology. A series of adjacent sections from above were stained with hematoxylin and eosin (H&E), dehydrated and coverslipped with DPX mounting medium. These sections were examined by two naive examiners for histopathological changes in tendons of TR18 and 18-week HRLF rats (n=3 each). Tendons were assessed for histopathological changes using the Bonar scale, which assesses 4 factors on a 4-point (0-3) scale: cell shape and cellularity, collagen organization, and vascularization (Cook et al, 2004). Each of these determinations was made in the flexor digitorum tendons at the level of the wrist joint, at 3 microscope fields/rat, in two to three separate sections per rat.

Statistical Analyses. All data are expressed as mean ± SEM. Grip strength was analyzed by 2-way ANOVA with the factors week and limb. Biochemical assays were analyzed using one-way ANOVAs. The Bonferroni method was used for post-hoc analyses, with results compared to NC and/or TR data; adjusted p values reported. A Kruskal-Wallis nonparametric test was used to determine differences in tendon pathology scores. A p value < 0.05 was considered significant. ANOVA results are shown in figures.

Results

Grip Strength declined progressively in the HRLF reach limb. Forearm grip strength was reduced compared to NC immediately after training and then persisted in the preferred reach limbs of HRLF rats (two way ANOVA: group $p<0.0001$, week $p<0.0001$, interaction $p=0.002$) (Fig. 1). Specifically, post hoc analysis showed that grip strength was significantly reduced, bilaterally, in TR0 and HRLF rats immediately after the initial training period. These declines continued in the reach limb throughout this 18 week study, compared to NC (See Fig. 1 for p values). In contrast, grip strength declines recovered with rest in trained-only rats (TR+Rest), and in the contralateral support limb of the HRLF rats.

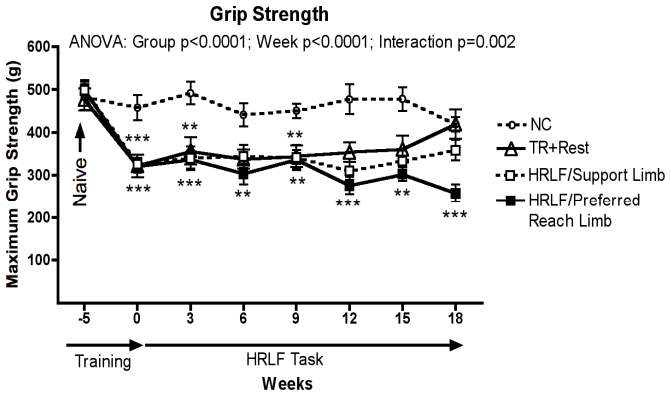

Figure 1. Grip strength in normal controls (NC), TR+Rest (trained only rats that rested for 18 weeks), and high repetition low force (HRLF) rats. Preferred reach limb and support limbs were examined. *:p<0.05, **p<0.01, and ***p<0.001, compared to NC.

Serum inflammatory analytes. Several inflammatory cytokines increased in the serum with performance of the HRLF task (Fig. 2). TNF-α and MIP3, were increased in serum in 6-week and 12-week HRLF rats, compared to NC rats (See Fig. 2 for p values). IL-10 was increased in serum only in 6-week HRLF rats, while MIP2 was increased in serum only in 12-week HRLF rats, compared to NC, TR0 and TR18 rats. Each resolved towards NC levels in 18-week HRLF. IL-12 was also increased in 18-week HRLF rats, compared to NC rats ($p<0.05$; data not shown).

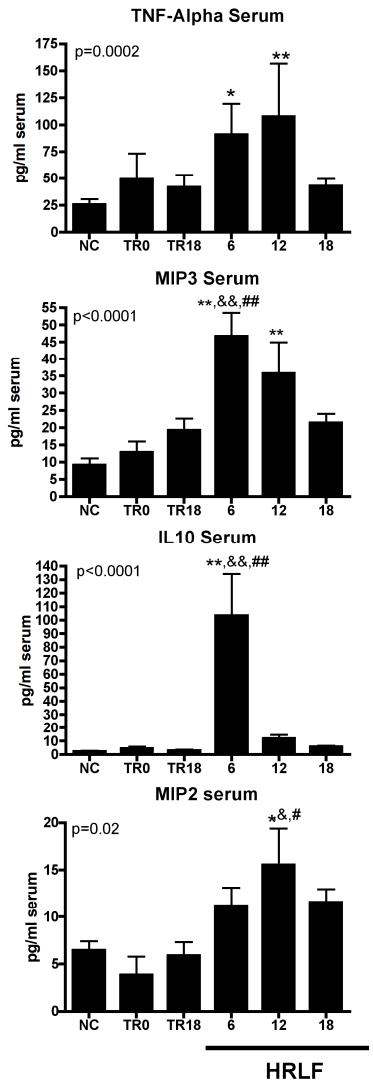

Figure 2. Serum TNF-α, IL-10, MIP3 and MIP2 in normal controls (NC), TR0 (rats euthanized immediately after training period), TR18 (trained-only rats that rested for 18 weeks), and high repetition low force (HRLF) rats that performed the task for 6, 12, or 18 weeks. *: p<0.05, **: p<0.01, compared to NC; &: p<0.05, &&: p<0.01, compared to TR0 rats; #: p<0.05, ##: p<0.01, compared to TR18 rats.

Tissue inflammatory cytokines. TNF-α had low-grade, but significant increases in the flexor digitorum muscle in 12-week HRLF reach limbs (Fig. 3). There were also clear but nonsignficant increases in the reach limb after training (in TR0 rats) and in the contralateral support limb of 12-week HRLF rats. TNF-α was increased seven-fold higher in flexor digitorum tendons than in muscles in 6- and 12-week HRLF reach limbs. IL-10, MIP2 and IL-12 were not significantly increased in flexor digitorum tendons or muscles, compared to NC.

Serum analyte of collagen degradation. Matrix metalloproteinase-2 (MMP2) was increased in serum of 18-week HRLF rats, compared to NC and TR18 rats (p<0.05 and p<0.01, respectively).

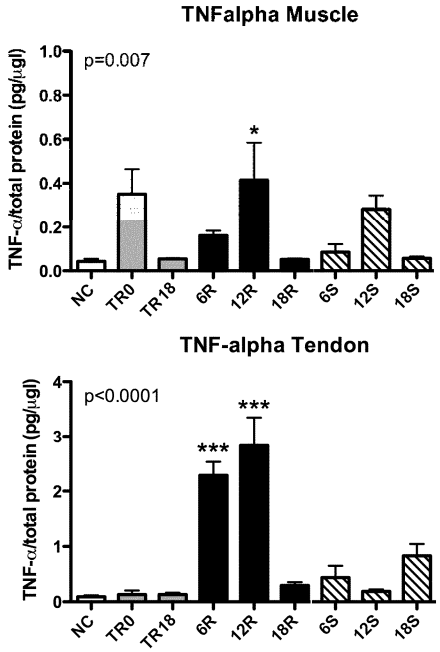

Figure 3. TNF-α, a key pro-inflammatory cytokine, levels in the flexor digitorum muscles and tendons of NC, TR0, TR18 and HRLF rats after 6, 12, and 18 weeks of task performance. R = reach limb; S = support limb*: p<0.05, **: p<0.01, compared to NC.

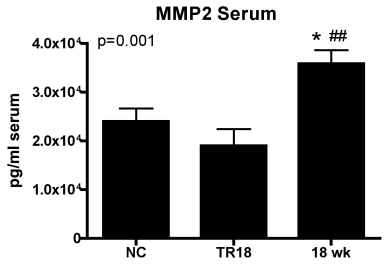

Figure 4. Serum MMP2 in NC, TR18 controls, and rats that performed the HRLF task for 18 weeks. Symbols as shown in Fig. 2.

Evidence of tendon degradation and fibrosis. Using ELISA, western blots and immunohistochemistry, we found increases in several degradative enzymes (MMPs) and fibrogenic proteins (TGFB1 and CTGF) in the flexor digitorum tendons of 18-week HRLF reach limbs. As shown in Fig. 5, MMP2 and TGFB1 increased in 18-week HRLF tendons, compared to TR18 tendons (p<0.05 and p<0.01, respectively). Immunohistochemical analysis showed that MMP-1, MMP-2, and MMP-13 were increased in both the epitendon and the endotendon of 18-week HRLF rats, compared to NC and TR18 tendons (data not shown). There were also increased TGFβ and CTGF immunopositive cells throughout the tendons and tendon sheaths, indicative of an ongoing fibroblast repair process (data not shown).

Tendon pathology was then analyzed histologically using the Bonar scoring method. The epitendon of 18-week HRLF rats was thickened, had increased cellularity, and an alteration in fibroblast cell shape, compared to TR18 rat epitendon (p<0.05; Fig. 6). The elongated shape of fibroblasts observed

in TR18 tendons, was lost in 18-week HRLF tendons, in which they appeared rounded.

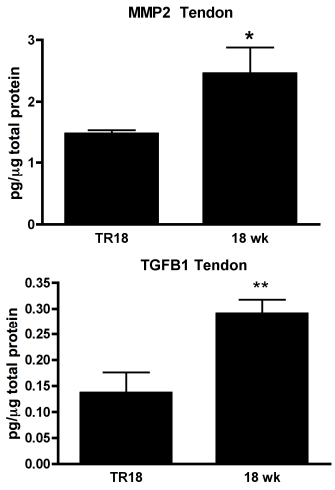

Figure 5. MMP2 and TGFB1 in the flexor digitorum tendons of TR18 and 18-week HRLF rats. *:p<0.05, **:p<0.01, compared to TR18.

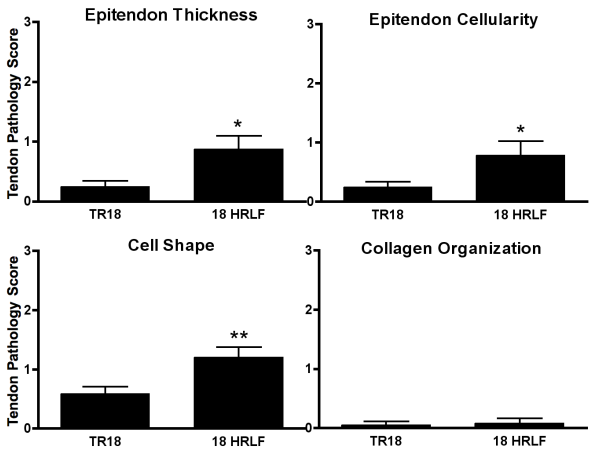

Figure 6. Bonar scoring data of tendon pathology.*:p<0.05 and **:p<0.01, compared to TR18.

Evidence of a low-grade increase of a fibrogenic protein, CTGF, in muscles. Using western blot analysis, we observed a small but significant increase in CTGF in reach limb flexor digitorum muscles in 18-week HRLF, compared to NC rat muscles ($p<0.05$; Fig 7).

Discussion

We observed persistent declines in forearm grip strength with long-term overuse activity at a moderate task level. These declines were associated initially with a low-grade inflammatory response in forearm flexor digitorum muscles (evidenced by a small but not significant increase in TNF-α in the muscle). As the task continued, serum levels of TNF-α rose significantly above baseline levels, an increase that was matched temporally by significant increases in TNF-α in forearm muscles and tendons. A cyclical inflammatory response was observed in the muscle. By week 18 of task performance, the serum inflammatory response had resolved. However, a biomarker of collagen degradation, MMP2, was significantly increased in serum at this later time point. The increase in serum MMP2 was matched temporally by many tissue changes indicative of degradative and fibrotic processes, including an increase in MMP2 in forearm tendons.

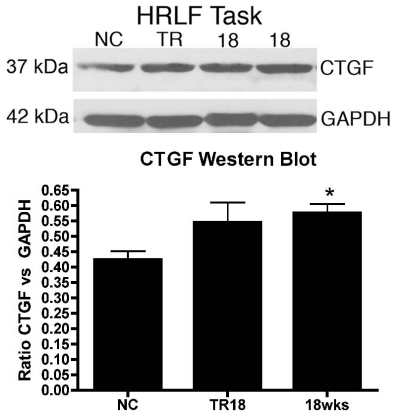

Figure 7. Western blot of muscles from NC, TR18 and 18-week HRLF forelimb muscles probed with anti-CTGF. GAPDH was used as a loading control. The ratio of CTGF/GAPDH is shown. The result of 3 experiments is shown.

Regarding the motor changes, declines were present immediately after the training period (TR0 time point of TR+Rest and HRLF rats). Grip strength resolved with rest in the trained-only rats and by week 12 in the support limbs of HRLF rats. This matches findings in a recent paper from our lab showing a reduction in grip strength after training in rats performing the HRLF task (Kietrys et al, 2011). The recovery of grip strength with rest in the TR+Rest rats is suggestive of tissue healing as a consequence of rest. The recovery of grip strength in the support limbs of HRLF rats may be due to adaptation of the tissues to the demands of providing support against the chamber wall. In contrast, the continued use of the reach limb to pull on the lever across the 18 weeks led to persistent grip strength declines with no sign of resolution.

The serum inflammatory cytokine response was low but still significantly increased for TNF-α, MIP 2 and MIP3 with performance of this moderate demand task. Several inflammatory cytokines tested were not above detectable levels and there was no increase after training. In fact, in this study, different and more sensitive ELISA assay kits were used than in our recent Xin et al, 2011 study. In that study, no changes in serum cytokines were detected in young adults rats that had performed the HRLF task for 12 weeks, although the significant increase in serum MIP2 levels were evident in that study as in this one. The levels of serum TNF-α, MIP2, and MIP3 here are similar to those observed in a prior study examining the effects of performance of a high repetition negligible force, food retrieval task (Barbe et al, 2008).

We also observed a low-grade tissue inflammatory response in musculotendinous tissues with long-term performance of this moderate demand reaching and lever-pulling task. This TNF-α inflammatory response was cyclical in nature in muscle, in that it was evident after the initial training session (albeit low grade), not present in 6-week

HRLF rats, but then reappeared in 12-week HRLF rats. Tendon seemed more effected, with increases after training (TR0), and in 6- and 12-week HRLF rats. However, TNF-α again resolved in 18-week HRLF. The initial peak of tissue inflammation after training is likely due to training-induced tissue injury. The resolution phase in trained animals and in the HRLF support limbs is due to tissue repair as a consequence of rest. In the HRLF task rats, the resolution of inflammation by week 12 is probably due to tissue adaptation to the demands of the task. However, the reappearance of the tissue inflammatory response thereafter suggests that tissue repair and growth processes did not keep pace with tissue degenerative processes.

The tissue inflammatory response was considerably lower in this study than in past studies from our lab in which rats performed tasks with higher force levels or with more fine-manipulative requirements (Fedorczyk et al, 2010; Barbe et al, 2008). We have also already shown correlations between increased inflammatory cytokines in forearm muscles and tendons and decreased forearm grip strength (Barbe et al, 2008; Coq et al, 2009). Thus, even though at low levels, the increased TNFalpha in muscles and tendons of HRLF reach limbs at 6 and 12 weeks likely contributed to grip strength declines in these same limbs.

In contrast to the low-grade inflammatory responses, collagen degradative changes were evident in both serum and forearm tissues. MMP2 was increased in serum and forearm tendons. We also observed increases in other MMPs (MMP1 and 13, data not shown) in 18-week HRLF reach limbs. Each of these MMPs is either a collagenase or a gelatinase that is involved in collagen catabolism; each increases in tendons after injury (De Mello Malheiro et al., 2009). The observed increase of three MMPs in the tendons support the presence of tendon degradative processes as a result of long-term performance of this moderate demand repetitive task.

We also observed increased TGFβ1 and CTGF using immunohistochemistry and western blot analysis (not all data shown). This observation is in line with those of studies showing that CTGF, collagen type 1, and TGFB1 increase under conditions of muscle overload or injury and are linked to tissue fibrosis (Kjaer, 2004; Abdelmagid, et al, 2012).Their increase in the tendons of 18-week HRLF task rats is more indicative of a fibrotic response than a regenerative repair response. These degenerative changes likely also contributed to grip strength declines observed. Our findings show that serum TGFβ1 should also be monitored in the future as an indicator of a fibrotic state, since it has been shown to be a sensitive serum biomarker of fibrogenic diseases (Neuman et al, 2012).

These findings show that even low demand tasks can induce tissue degradative changes if the work is performed for long periods of time. Also, the temporal association of grip strength declines with both the low-grade tissue inflammatory cytokine response and the degenerative tendon changes support a contribution from each process to functional declines occurring with overuse. Lastly, one serum biomarker, MMP2, was pinpointed as a marker of underlying tendon degenerative changes. Serum levels of TGF-beta and CTGF levels were not tested in this study, but may also serve as biomarkers of tissue degradative processes induced by long-term performance of repetitive tasks.

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References:

Abdelmagid SA, Barr AE, Rico M, Amin M, Litvin J, Popoff SN, Safadi FF, Barbe MF. Performance of Repetitive Tasks Induce Decreased Grip Strength And Increased Fibrogenic Proteins in Skeletal Muscle: Role of Force and Inflammation. PLoS ONE 7(5): e38359. doi:10.1371/journal.pone, 2012.

Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF. Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. J Orthopedic Res 21(1): 167-176, 2003.

Barbe MF, Elliott MB, Abdelmagid SM, Amin M, Popoff SN, Safadi FF, Barr AE. Serum and tissue cytokines and chemokines increase with repetitive upper extremity tasks. Journal of Orthopaedic Research, 26(10), 1320-1326, 2008.

Bureau of Labor Statistics. Nonfatal occupational injuries and illnesses requiring days away from work 2010. Released 10:00 a.m. (EST) Wednesday, November 9, 2011; USDL-11-1612.

Carp SJ, Barbe MF, Winter KA, Amin M, Barr AE. Increased Cytokines and C-reactive protein in human serum are associated with severity of musculoskeletal disorders from Overuse. Clin Sci (Lond). 112(5):305-14, 2007.

Cook JL, Kiss, ZS, Khan KM, Purdam, CR, Webster KE. Anthropometry, physical performance, and ultrasound patellar tendon abnormality in elite junior basketball players: a cross-sectional study. British journal of sports medicine, 38(2), 206-209, 2004.

Coq JO, Barr AE, Strata F, Kietrys D, Merzenich MM, Byl NN, Barbe MF. Peripheral and central changes combine to induce motor behavioral deficits in a moderate repetition task. Exp Neurol. 220(2):234-245, 2009.

De Mello Malheiro OC, Giacomini CT, Justulin, LA, Delella FK, Dal-Pai-Silva M, Felisbino, SL. Calcaneal tendon regions exhibit different MMP-2 activation after vertical jumping and treadmill running. Anatomical record, 292(10), 1656-1662, 2009.

Garner BC, Stoker AM, Kuroki K, Evans R, Cook CR, Cook JL. Using animal models in osteoarthritis biomarker research. J Knee Surg. 24(4):251-64, 2011.

Fedorczyk JM, Barr AE, Rani S, Amin M, Gao H, Amin S, Litvin J, Barbe MF. Exposure dependent increases in IL-1beta, substance P, CTGF and tendinosis in flexor tendons with upper extremity repetitive strain injury. J Orthop Res. 28(3):298-307, 2010.

Kietrys DM, Barr AE, Barbe MF. Exposure to Repetitive Tasks Induces Motor Changes Related to Skill Acquisition and Inflammation in Rats. J Mot Behav. 43(6):465-76, 2011.

Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol Rev. 84(2), 649-698, 2004.

Neuman MG, Schmilovitz-Weiss H, Hilzenrat N, Bourliere M, Marcellin P, Trepo C, Mazulli T, Moussa G, Patel A, Baig AA, Cohen L. Markers of inflammation and fibrosis in alcoholic hepatitis and viral hepatitis C. Int J Hepatol. 231210, 2012.

Rechardt M, Shiri R, Matikainen S, Viikari-Juntura E, Karppinen J, Alenius H. Soluble IL-1RII and IL-18 are associated with incipient upper extremity soft tissue disorders. Cytokine 54(2):149-53, 2011.

Xin, DL, Harris MY, Wade CK, Amin M, Barr AE, Barbe MF. Aging enhances serum cytokine response but not task-induced grip strength declines in a rat model of work-related musculoskeletal disorders. BMC Musculoskeletal Disorders 12: 63, 2011.