



# High magnitude cyclic load triggers inflammatory response in lumbar ligaments

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## ABSTRACT

**Background:** Cumulative trauma disorder is commonly reported by workers engaged in prolonged repetitive/cyclic occupational activities. Recent experimental evidence confirms that relatively short periods of cyclic lumbar flexion at high loads result in substantial creep of viscoelastic tissues, prolonged periods of its recovery to baseline together with a neuromuscular disorder and exposure to instability. The biochemical process associated with the creep and neuromuscular disorder are not well explored. The purpose of the study is to identify the ligaments as one of the organs of failure and an acute inflammation as the result of failure as a preliminary step in the development of chronic inflammation that might lead to cumulative trauma disorder elicited by high magnitude cyclic loads.

**Methods:** The lumbar spine of anaesthetized cats was subjected to cyclic flexion loading at high magnitudes for six periods of 10 min each with 10 min rest in between followed by 7 h rest. Lumbar displacement was monitored throughout. Supraspinous ligaments from L-3/4, L-4/5, L-5/6 and unloaded T-10/11 were removed at the end of testing and assessed using mRNA expression for cytokine (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , TGF $\beta$ ). Cytokines expression in the lumbar ligaments were statistically compared to their self control in the unloaded thoracic ligament. The creep developed during the loading and its recovery during the 7 h rest was calculated.

**Findings:** The mean creep developed during the loading period reached 57.3% recovering to a residual value of 25.5% at the end of the 7 h rest. Increase in cytokine expression was seen in all lumbar ligaments with statistical significance in the L-4/5 and L-5/6 levels.

**Interpretation:** The results confirm that prolonged high magnitude cyclic loading of the lumbar spine in flexion–extension elicits substantial residual creep together with significant increases in cytokines expression, consistent with an acute inflammation, several hours post loading. Further exposure to cyclic loading over time may result in conversion to chronic inflammation.

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## 1. Introduction

Cumulative trauma disorder (CTD) is commonly reported by workers exposed to long-term cyclic/repetitive occupational activities and in the lumbar spine it is diagnosed with the presence of persistent low back pain, weakness, limited range of motion and spasms/stiffness of paraspinal muscles. It is a chronic disorder that severely impacts the workforce, health care and economy. The epidemiology predicts the occurrence of CTD in individuals exposed to long-term cyclic loads of high magnitude, fast motion, insufficient rest and multiple repetitions (Silverstein et al., 1986; Punnett and Wegeman, 2004; Hoogendoorn et al., 2000; Marras, 2000). Recent biomechanical and neurophysiological evidence confirmed the epidemiology, demonstrating sustained creep/laxity in the lumbar

viscoelastic tissues, spasms and hyperexcitability of the multifidi, and neuromuscular compromise (compensation) of lumbar (in)stability following cyclic work in the in vivo feline model (Navar et al., 2006; Hoops et al., 2007; Le et al., 2007; Lu et al., 2004, 2008; Solomonow et al., 2008), normal human subjects (Li et al., 2007; Olson et al., 2004, 2006, 2009; Dickey et al., 2003; Shin and Mirka, 2007; Little and Khalsa, 2005; Karajcarski and Wells, 2006; Granata et al., 1999, 2005) and low back pain patients (van Dieen et al., 2003). Low magnitude cyclic loads applied at low frequency, low number of repetitions and with sufficient rest, however, do not trigger the disorder. Cyclic loading, therefore, can be separated into high and low risk categories.

Very little is known relative to the organ of failure and the process causing the failure giving rise to the CTD. Solomonow et al. (2003) offered limited preliminary data which indicated a many fold increase in neutrophil density in feline lumbar ligaments, several hours following exposure to high magnitude cyclic loads. It may be reasonable to assume that high risk cyclic loading may give rise to excessive creep (Solomonow et al., 1999; McGill and Brown,

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1992) and the associated micro-damage in the viscoelastic tissues (Fung et al., 2009; Woo et al., 1982), which in turn elicit inflammatory response (Leadbetter, 1990).

It is hypothesized that prolonged exposure to high magnitude cyclic loads induces sustained creep and micro-damage in the collagen fibers of the posterior lumbar ligaments which, in turn, trigger a pro-inflammatory response. The objective of this report, therefore, is to demonstrate that exposure of the lumbar spine to anterior flexion–extension under high magnitude cyclic loads elicits sustained creep and significant increase in pro-inflammatory cytokines.

## 2. Methods

### 2.1. Preparation

Eleven (11) adult cats, with an average weight of  $3.06 \pm 0.44$  kg, were used in this study. They were anesthetized with 60 mg/kg chloralose, according to a protocol approved by the Institutional Animal Care and Use Committee. A superficial skin incision overlying the lumbar spine was made to expose the dorso-lumbar fascia, and an S-shaped stainless steel hook made of 1.5-mm-diameter rod was applied around the supraspinous ligament between L<sub>4</sub> and L<sub>5</sub>. The preparation was then positioned in a rigid stainless steel frame and the lumbar spine was isolated by means of two external fixators which were applied to the L<sub>1</sub> and L<sub>7</sub> posterior process. The external fixation was intended to limit the elicited flexion to the lumbar spine and to prevent interaction of thoracic and sacral and/or pelvic structures, but not to prevent any motion. A schematic of the set-up as well as radiological verification of flexion–extension function resulting from the applied load are available in our previous reports (Solomonow et al., 1999; Williams et al., 2000).

### 2.2. Instrumentation

The S-shaped stainless steel hook inserted around the L<sub>4-5</sub> supraspinous ligament was connected to the crosshead of the Bionix 858 Material Testing System (MTS, Minneapolis, MN), in which a load cell was located. The load was applied through the MTS actuator with a computer-controlled loading system and monitored continuously along with the vertical displacement of the actuator. Displacement was monitored by an internal sensor in the actuator. The load cell and displacement outputs of the Bionix 858 MTS were also sampled into the computer at 1000 Hz. Under such loading condition, the lumbar spine underwent anterior flexion–extension while straining the lumbar supraspinous ligaments.

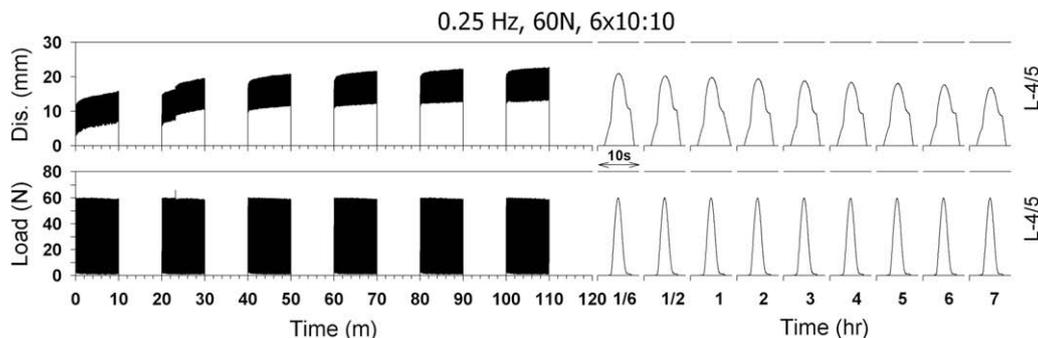
### 2.3. Protocol

A pre-load of 1 N was applied just prior to each single period of cyclic loading in order to produce a standard baseline across all preparations. A set of six 10 min cyclic loading periods at 0.25 Hz and 60 N peak, each followed by 10 min rest was applied for a cumulative cyclic loading period of 60 min. The following recovery phase consisted of 7 h of rest at no load, during which single test cycles of 60 N peak load at 0.25 Hz were applied. The single cycles were applied at 10 min, 30 min and 60 min following termination of the cyclic loading period and then once every hour. Overall, nine test cycles were applied during the 7 h recovery period. Fig. 1 (bottom trace) shows the schematic of the cyclic loading. Load and displacement were recorded throughout the protocol. Sixty (60) N was established by previous work (Solomonow et al., 1998) to constitute a non-injurious high load, near the 90th percentile of the maximal load of specimen of the same age and weight. Loads of 70 N resulted in partial ligament rupture in 10% of the group tested and loads of 80 N ruptured 90% of the ligaments in the group tested. The 60 N load, therefore, was considered as a high, non-injurious load within the physiological range of the ligament. The cyclic frequency was selected to be 0.25 Hz based on prior measurements in normal humans performing deep flexion/extension at normal, comfortable pace. At the end of the 7 h recovery period, the supraspinous ligaments of the L-3/4, L-4/5 and L-5/6 were harvested from the specimen for cytokines analysis. For purposes of comparison, the supraspinous ligament of T-10/11 from each preparation was also removed for analysis as self control since it was not subjected to the cyclic load or any associated motion. Comparison of lumbar ligaments to un-stimulated thoracic ligament as self control was scientifically advantageous. This, however, requires confirmation that baseline/un-stimulated cytokines level in different ligaments of the same specimen is approximately the same. For validation of this assumption, a control group of animals was tested and the validation is described in Appendix A.

### 2.4. Cytokines analysis

#### 2.4.1. RNA extraction and preparation of cDNA

Ligaments were flash frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  then powdered in a laboratory ball mill (Mikro-Dismembrator S, Sartorius BBI Systems, Inc., Bethlehem, PA). RNA was extracted and purified from the powdered ligaments using the RNeasy Lipid Tissue Mini Kit according to manufacturer's directions (QIAGEN, Valencia, CA). The procedure included an on-column DNase step. The average concentration, purity, and yield of RNA extracted from all ligaments were 120 ng/ $\mu\text{l}$ , 2.07 ( $\lambda$  260/280 nm), and 3.7  $\mu\text{g}$ , respectively. Complementary DNA (cDNA) was prepared from



**Fig. 1.** Typical recording of the six sets of 10 min cyclic load spaced by 10 min rest is shown on the bottom and the corresponding flexion displacement of the lumbar spine is shown on the top. Note the creep developing during the six loading periods and its recovery during the 7 h of following rest.

1  $\mu\text{g}$  of RNA isolated from each sample using the HC cDNA RT kit with RNase inhibitor (Applied Biosystems, Foster City, CA).

#### 2.4.2. Quantitative real-time PCR

Expression of gene targets was measured using real-time RT-PCR. Primers and probes for IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , and TGF $\beta$  were designed with the assistance of the Primer Express sequence detection software (Applied Biosystems, Foster City, CA). Cytokines are soluble proteins that respond to ligament injury by binding to their receptors and initiating a cellular response (Smith and Humphries, 2009). Interleukin-1 (IL-1) is a strong pro-inflammatory cytokine that works both directly and indirectly. IL-1 induces B-cell differentiation and acute phase proteins (Gauldie et al., 1987). It also drives extracellular matrix destruction by increasing degradative enzymes such as matrix metalloproteinases (Tsuzaki et al., 2003). In addition, IL-1 stimulates fibroblasts to express and secrete other inflammatory cytokines including IL-6 (Van Damme et al., 1987; Guerne et al., 1989). The action of TNF $\alpha$  is similar to that of IL-1, but is thought to be less potent in most tissues. The action of IL-6 is typically pro-inflammatory during the acute phase by inducing the production of acute phase proteins (some distinct from those produced by IL-1 and TNF $\alpha$ ) and can also promote B and T cell functions and stimulate the secretion of immunoglobins (Lipsky, 2006; Guerne et al., 1989). IL-8 is considered a chemokine because it attracts neutrophils to the site of inflammation (Namen et al., 1988), for example, in inflammatory joint disease (Verburgh et al., 1993). The remaining cytokine measured in this study, TGF $\beta$ , is a multifunctional cytokine that acts in normal physiology and pathology. It may act in potentially contradictory ways depending on tissue and pathology (Massague and Gomis, 2006). In inflammation, TGF $\beta$  can recruit B and T cells, but can also lower the immune response and promote extracellular matrix production.

TaqMan probes (Applied Biosystems, Foster City, CA) with 5'-label of 6-carboxy fluorescein and 3'-label of 6-carboxy-tetramethylrhodamine. Fifty micro-liter reaction mix containing TaqMan Universal Master Mix, forward and reverse primers, probes, RNase-free water, and 1  $\mu\text{g}$  of the template cDNA was amplified for each target using an ABI prism 7500 sequence detector (Applied

Biosystems, Foster City, CA) with an initial melt at 95  $^{\circ}\text{C}$  for 10 min followed by 40 cycles of amplification at 95  $^{\circ}\text{C}$  for 15 s and 60  $^{\circ}\text{C}$  for 1 min. Real-time data acquisition and analysis were performed using Ct values in which mRNA levels for each gene were normalized to the corresponding expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A standard curve was generated for each gene using the fluorescent data from the 10-fold serial dilutions of the amplicon that matched the specified primers. The standard curves for each gene did not differ significantly between experiments meaning that normalized values could be compared directly between experiments.

#### 2.5. Statistical analysis

Normalized cytokine levels taken from ligaments harvested from the loaded region (L<sub>3-4</sub>, L<sub>4-5</sub>, L<sub>5-6</sub>) were compared to normalized cytokine levels taken from a ligament harvested outside of the loaded region (T<sub>10-11</sub>) in each specimen as a self control (see Appendix A for validation). A mixed-factor analysis of variance with specimen as a random variable was used for this comparison, allowing each specimen to serve as its own control. The fixed independent variable in the statistical model was ligament level (T<sub>10-11</sub>, L<sub>3-4</sub>, L<sub>4-5</sub>, L<sub>5-6</sub>), and the dependent variables were the five normalized cytokine values (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , TGF $\beta$ ). Distributions from all cytokines displayed a right-tailed shape; therefore, each was transformed to a normal distribution using a natural log before analyses. Upon significant effect of ligament level, post hoc pairwise comparisons were performed using Student's *t*-tests. Alpha was set to 0.05 for all statistical tests.

### 3. Results

Fig. 1 displays a typical loading sequence with six work/rest phases followed by a seven hour rest period as well as the associated displacement recorded from one preparation.

Fig. 2 provides the mean  $\pm$  SD of the displacement from the 11 preparations and the associated mean creep during the loading and following 7 h rest period. The initial mean displacement was

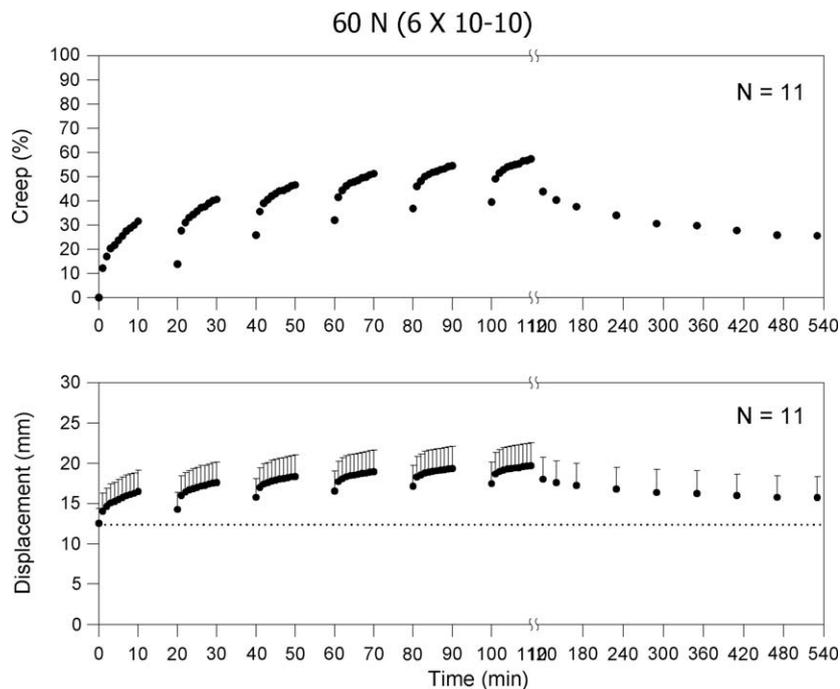


Fig. 2. The mean  $\pm$  SD of the displacement from all specimens during the cyclic loading and the following 7 h recovery period is shown on the bottom whereas the top shows the associated creep that developed within the viscoelastic tissues.

12.52 mm and gradually increased during the first 10 min of cyclic loading to 16.47 mm with a corresponding creep of 31.5%. It partially recovered during the first 10 min rest to 14.2 mm (13.7% creep) and then further increased during each additional 10 min loading reaching a final mean value of 19.69 mm with a corresponding mean creep of 57.3%. During the 7 h rest period the displacement gradually decreased reaching a residual mean value of 15.71 mm with a corresponding residual mean creep of 25.5%. Full

recovery of the displacement or the creep was not observed in any of the preparations.

Fig. 3 displays the data for each of the cytokines measured in each of the three lumbar ligaments as well as the self control T-10/11 ligament at the end of the 7 h rest period. The main effect of ligament level was significant ( $P < 0.001$ ) for all cytokines. Pair-wise comparisons revealed similar results for all cytokines when comparing the loaded lumbar levels to the unloaded thoracic

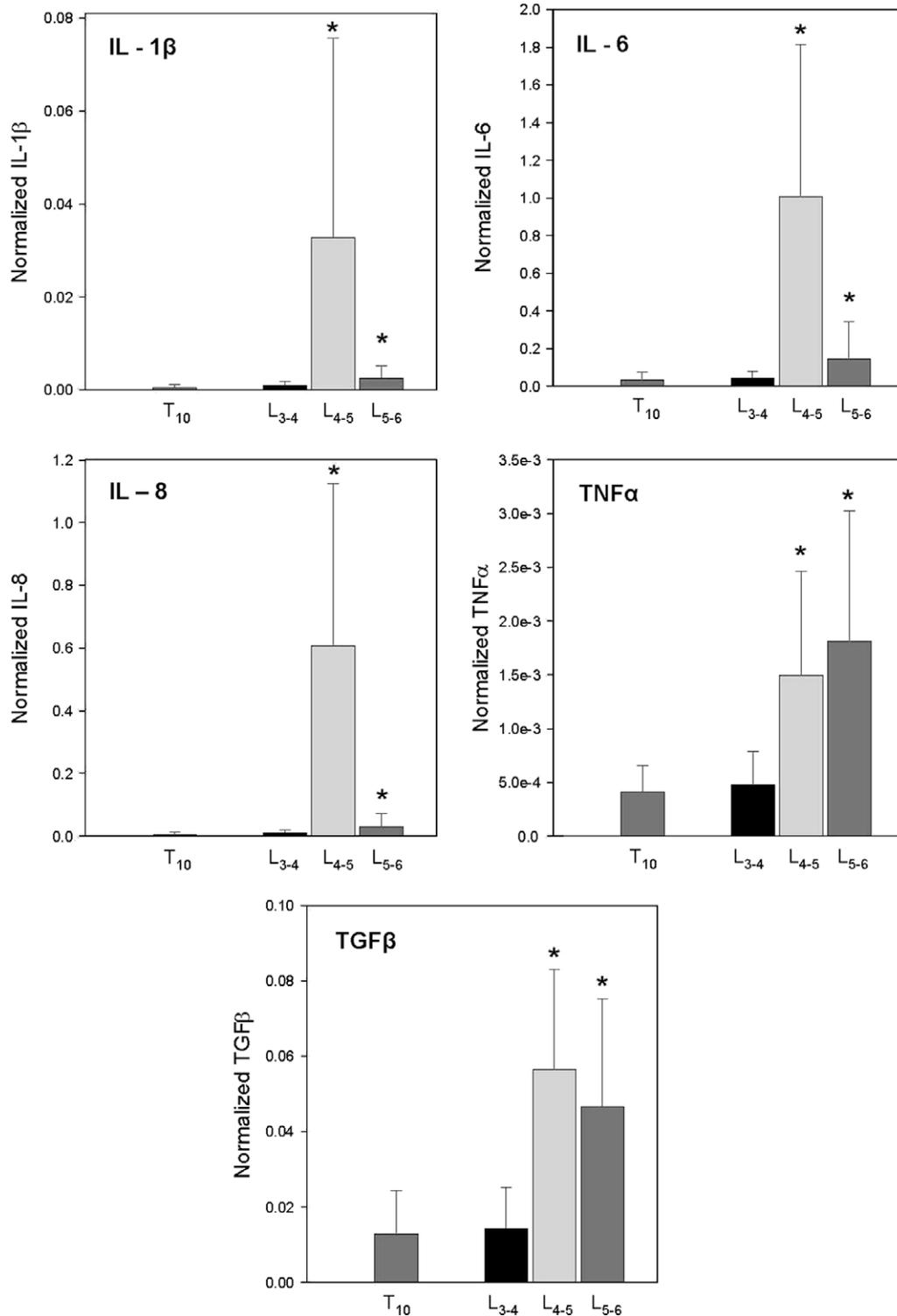


Fig. 3. Normalized concentration of the IL-1B, IL-6, IL-8, TNFα and TGFβ associated with the lumbar levels T-10/11 (used as self control) and L-3/4, L-4/5 and L-5/6. (\*) indicates that a significant difference exists between that lumbar level and the corresponding T-10/11 level.

**Table 1**

Mean  $\pm$  SD of multiplicative within-specimen increases for each lumbar level relative to T-10 control.

Cytokine	Loaded level		
	L <sub>3-4</sub>	L <sub>4-5</sub>	L <sub>5-6</sub>
IL-1 $\beta$	3.6 $\pm$ 4.5	134.0 $\pm$ 160.3	11.5 $\pm$ 15.8
IL-6	3.5 $\pm$ 4.3	85.2 $\pm$ 99.5	16.3 $\pm$ 37.6
IL-8	3.8 $\pm$ 3.3	285.1 $\pm$ 347.2	13.4 $\pm$ 18.6
TNF $\alpha$	1.4 $\pm$ 1.3	6.7 $\pm$ 9.4	5.9 $\pm$ 7.1
TGF $\beta$	1.3 $\pm$ 0.9	6.9 $\pm$ 6.3	5.2 $\pm$ 5.4

level: only L<sub>4-5</sub> and L<sub>5-6</sub> were significantly higher than T<sub>10-11</sub> while L<sub>3-4</sub> did not demonstrate any differences from T<sub>10-11</sub>. Pair-wise comparisons also revealed higher levels of interleukin cytokine levels in L<sub>4-5</sub> compared to L<sub>5-6</sub>. Expressed as multiples of the T-10/11 control, IL-1 $\beta$  was 134.0 times higher than control in L<sub>4-5</sub> while only 11.5 times higher than control in L<sub>5-6</sub>. IL-6 was 85.2 times higher than control in L<sub>4-5</sub> while only 16.3 times higher than control in L<sub>5-6</sub>. IL-8 was 285.1 times higher than control in L<sub>4-5</sub> while only 13.4 times higher than control in L<sub>5-6</sub>. Contrary to the interleukin response, TNF $\alpha$  and TGF $\beta$  levels showed no differences between L<sub>4-5</sub> and L<sub>5-6</sub>. TNF $\alpha$  was 6.7 times and 5.9 times higher than control in L<sub>4-5</sub> and L<sub>5-6</sub>, respectively. TGF $\beta$  was 6.9 times and 5.2 times higher than control in L<sub>4-5</sub> and L<sub>5-6</sub>, respectively. Please refer to Table 1 for a full summary.

#### 4. Discussion

The primary findings of this investigation consist of the identification of significant increase of cytokines expression in the lumbar supraspinous ligaments together with substantial residual creep 7 h after being subjected to high magnitude cyclic loads, eluding to the presence of acute inflammation.

Substantial creep (31%) was developed in the lumbar spine during the first 10 min loading session and only partially recovered over the following 10 min rest. That pattern continued throughout the remaining five loading/rest sessions, manifesting with cumulative creep of 57%. The viscoelastic tissues were obviously strained by the prolonged cyclic loading resulting in substantial laxity. The seven hours of rest did not allow for full recovery of the creep, leaving a residual value of 25%. The residual creep value may be considered as an indication of accumulated micro-damage in the collagen fibers (Wang and Ker, 1995; Schechtman and Bader, 2002; Fung et al., 2009), which may take substantial time to resolve (Solomonow et al., 2000). Fig. 2 demonstrates that the exponential recovery of the displacement and creep reached their asymptotic phase by the 7th hour of rest, further confirming that a fast additional recovery of creep is not expected within the next several hours. Most likely, the micro-damage developed in the collagen fibers of the ligaments has to be addressed and repaired by the ensuing acute inflammation (Leadbetter, 1990) before the ligaments can be restored to their normal resting length and function. Indeed, a model fitted to the creep/displacement data of the set-up described here and exposed to similar load protocol points out that 48–72 h may be required for full recovery to near resting length (Solomonow et al., 2000). This may further support that a spontaneous recovery by a short acute inflammation may be required to restore tissue resting length and stiffness properties.

The increase in the specific cytokine genes expressed in the strained ligaments, together with our demonstration of over 100 $\times$  increase in neutrophil concentration in similarly loaded supraspinous ligaments (Solomonow et al., 2003), indicate an acute inflammatory response. Neutrophil density increase of such magnitude is a standard pathological diagnostic protocol for iden-

tifying inflammation. Therefore, the increase in pro-inflammatory cytokines associated with standard confirmation of inflammation fully asserts the cumulative injurious effect of repetitive high magnitude loads on lumbar ligament.

This study reports the mRNA expression of five cytokines at a single time point. Due to limited tissue, cytokine protein has not been measured to confirm translation and secretion. However, considering the extremely high expression of interleukins-1, -6, and -8 (85–285-fold over within-animal control), functional activity of these cytokines in this model for the acute phase of repetitive injury seems likely. Because the activity of these cytokines perpetuate the inflammatory response through their effects on fibroblasts (to produce more cytokines and tissue degrading enzymes) and on inflammatory response cells (recruitment, maturation, and synthesis), inflammation could be sustained beyond the initial injury particularly in the continued presence of the original inflammatory stimulus (cumulative loading). On the other hand, removal of this stimulus (discontinue loading, i.e., rest) may restore cytokine expression to pre-inflammation levels. It should be noted, therefore, that the results point out that high load magnitudes applied repetitively within 2 h can induce an acute inflammation with the ability to perpetuate itself. Workers exposed to daily ongoing work with high loads, therefore, will continue to aggravate the acute inflammation to a point where it may convert to chronic inflammation, tissue degeneration and permanent disability defined by the CTD (Leadbetter, 1990).

Pro-inflammatory responses in ligaments and tendons in the extremities of animal models exposed to prolonged repetitive stimulation were described before (Soslowsky et al., 2000; Barbe et al., 2003). It is important to note that the lumbar ligaments are substantially different from the ligaments of the extremities. The physiologic strain of the human ACL, for example, is 6–7% (Renstrom et al., 1986), whereas the mid-lumbar supraspinous ligaments' physiological strain is 24%, with a range of up to 42% in humans (Panjabi et al., 1982). The physiological strain of the same lumbar ligaments in the feline is nearly identical (Williams et al., 2000) to that found by Panjabi et al. in humans. It should also be noted that the fibers undergo re-orientation during flexion in this physiological range (Adams et al., 1980). Despite the ability of the lumbar ligaments to undergo a 4–5-fold larger strain within the physiological range, they do develop significant creep, apparent micro-damage and an acute inflammatory response when subjected to high risk repetitive loads. It seems that the prolonged exposure to repetitive stimulation is the inflammation triggering component while the physiological strain properties of specific viscoelastic tissues play a secondary but not unimportant role. In conjunction with the above issues, flexion–extension loading was chosen such that the posterior viscoelastic tissues will be stretched as opposed to a loading mode of compression which does not engage the ligaments (Adams et al., 1980). Furthermore, while only the supraspinous ligament was assessed for cytokines expression in this study, it is possible that other viscoelastic tissues developed inflammatory responses. The flexion–extension loading surely stretched the intraspinal and posterior longitudinal ligament, the facet capsule, the dorso-lumbar fascia as well as deformed the discs of the lumbar spine.

It should be noted that the supraspinous ligaments of L-3/4 demonstrate a 1.3–3.8-fold, yet statistically non-significant, increase in cytokines expression relative to the un-stimulated T-10/11 control, whereas the ligaments from the L-4/5 and L-5/6 levels did show significant increases. The absence of significance of the cytokines increase in the L-3/4 supraspinous ligaments post loading is explained by the physiological strain of the ligament at each lumbar level. The mean physiological strain of the L-3/4 supraspinous ligament is 21.3%, for the L-4/5 is 23.6% and for the L-5/6 ligament is 14.4% in the feline (Williams et al., 2000). It

seems that normally the supraspinous ligament's physiological strain is peaked at the L-3/4 and L-4/5 lumbar levels and substantially decreases for the L-5/6 level. Therefore, the loading applied and its associated strain and creep had much more impact on the L-5/6. This may have elicited more micro-damage in this level compared to the L-3/4 level that could accommodate more strain without damage from the applied load. The focal point of the stimulation was at the L-4/5 level and that supraspinous ligament was directly stimulated and expected to be fully exposed and develop micro-fractures. Overall, one can assume that the supraspinous ligament of the L-3/4 level was strained mildly relatively to its large physiological strain whereas the ligament at L-5/6 was strained well into its much smaller physiological range and therefore sustained substantial and repeated micro-fractures by the same load. Therefore, acute inflammation could be expected in the L-4/5 and L-5/6 but not in the L-3/4 level.

Finally, potential damage to the supraspinous ligament of L-4/5 from artefactual injury from the hook used for loading should be excluded. The hook was deliberately inserted around the ligament, not through it, with 2 mm of soft tissue buffer between the hook and the ligament. The intraspinal ligament, however, was penetrated by the hook but its tissue was excluded from harvesting for the mRNA analysis. Furthermore, increases in cytokines were observed in the L-3/4 and L-5/6 which were 4–5 cm away (in rostral and caudal directions, respectively) from the hook, excluded from any potential injury and indirectly stimulated by the flexion–extension loading. This confirms that the repetitive, high magnitude loading was the stimulus responsible for the cytokines increase.

Based on the results presented, the following conclusions can be made for the feline model stimulated with the described protocol: Prolonged exposure of the lumbar spine of an in vivo feline to high magnitude cyclic loads elicit a significant increase in pro-inflammatory cytokines consistent with an acute inflammation within several hours post work. Substantial residual creep is simultaneously observed in the lumbar spine, several hours post work, eluding that significant micro-damage in the viscoelastic tissues is the triggering source of the significant increase in pro-inflammatory cytokines.

The significantly elevated pro-inflammatory state triggered by repetitive flexion loading in this study *can be* a prelude to further complications if the inflamed lumbar spine is continued to be exposed to repetitive loading on a daily basis for a long period, allowing the conversion to a chronic inflammation, degeneration of the viscoelastic tissues into fibrous, non-functional tissue and the associated mechanical and neuromuscular disorders and loss of function.

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## Appendix A

Nine (9) adult cats served as a control group for validating that baseline cytokines level in different lumbar and thoracic ligaments is the same. Animals were anaesthetized in the same manner as described in the methods section and the supraspinous ligaments from T-10/11, L-3/4, L-4/5 and L-5/6 were immediately dissected out (without any loading) and sent for cytokines analysis in the same way described in methods. Similarly, the statistical analysis comparing cytokines in T-10/11 to that in the three lumbar ligaments was identical. The results show that the ligaments harvested from the control group showed no effect of vertebral level across

all cytokines. Results for effect of ligament level were as follows: IL-1 $\beta$  ( $P = 0.669$ ), IL-6 ( $P = 0.105$ ), IL-8 ( $P = 0.237$ ), TNF $\alpha$  ( $P = 0.544$ ), and TGF $\beta$  ( $P = 0.543$ ). This, therefore, validated that comparison of the stimulated (loaded) group to their unloaded self control T-10/11 ligament is appropriate.

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