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Basic Science

High-frequency loading of lumbar ligaments increases proinflammatory cytokines expression in a feline model of repetitive musculoskeletal disorder

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Abstract

BACKGROUND CONTEXT: Cumulative (repetitive) lumbar disorder is common in the workforce, and the associated epidemiology points out high risk for lifting heavy loads, performing many repetitions, and performing movements at high velocity. Experimental verification of viscoelastic tissue degradation and a neuromuscular disorder exist for cyclic work under heavy loads. Experimental validation for a disorder because of cyclic loads under high-velocity movement is missing.

PURPOSE: Obtain experimental verification that high-velocity lumbar flexion-extension results in significant increase of proinflammatory cytokines in the viscoelastic tissues.

STUDY DESIGN: Laboratory experiments using two in vivo feline model groups subjected to cyclic flexion-extension at low and high velocity.

METHODS: Seven hours after cumulative 60 minutes of cyclic flexion-extension at moderate load of 40 N and 0.25 Hz for first group and 0.5 Hz for the second group, the supraspinous ligaments of L3–L4 to L5–L6 were harvested and subjected to cytokines (interleukin [IL]-1β, IL-6, IL-8, tumor necrosis factor-α, and transforming growth factor-β1) analysis. Two-way mixed model analysis of variance with a post hoc analysis were used to assess any significant differences ($p<.05$) in cytokines expression level between the two groups as well as main effect and interaction with lumbar levels.

RESULTS: Expression levels of the five cytokines were significantly increased in the group subjected to the high-frequency loading.

CONCLUSIONS: Exposure of the lumbar spine to high-velocity flexion-extension triggers a significant increase in proinflammatory cytokines, indicating pronounced changes consistent with an acute inflammation. Further exposure to activity over prolonged periods may trigger chronic inflammation and tissue degeneration as the source of cumulative lumbar disorder.

Keywords: Cumulative disorder; Cytokines; Spine; Lumbar; Ligaments

Introduction

Cumulative trauma disorder (CTD), diagnosed with pain, weakness, limited range of motion, and stiffness of surrounding muscles/joints is common to workers and athletes exposed to repetitive/cyclic physical activity over extended periods of time [1]. The epidemiology identified several risk factors of which, high movement velocity (or high frequency of repetition) is a prominent one [2–5]. Recent research supports the hypothesis that repetitive or cyclic activity induces creep [6–10] and microdamage

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[11–13] in viscoelastic tissues (ligaments, discs, facet capsule, and dorsolumbar fascia). As the microdamage becomes severe enough, an acute inflammation [14–17] sets in the viscoelastic tissues, triggering spasms, pain, and sustained hyperexcitability of muscles that support and protect the joint [18–21]. Further exposure to cyclic activity may convert the acute inflammation into chronic inflammation, viscoelastic tissue remodeling, and eventual degeneration [14]. The neuromuscular disorder components were also observed in experimental humans [22–24] and low back pain patients [25,26].

Lumbar ligaments are unusual as they display substantially larger physiological strain [27,28] compared with extremity ligaments. Large increases in the velocity of movement, however, can still develop high tension in the lumbar tissues [29–32] and increase the microdamage in the collagen fibers and pose a substantially higher risk for inflammatory conditions. The effect of increased frequency of repetitive/cyclic movement on the development of proinflammatory conditions in lumbar ligaments was not previously explored and is the objective of this report.

We hypothesize that increased frequency of anterior flexion-extension of the feline lumbar spine will trigger significantly higher proinflammatory cytokines expression in the supraspinous ligaments compared with flexion-extension at lower frequency at the same peak load. The insight gained from such research can provide biomechanical and biological validation of the epidemiology, assist in justifying the design of preventive work protocols or medical treatments, and also identify the viscoelastic tissues as the organ of failure, via inflammation, in CTD.

Methods

Preparation

Twenty-one adult cats, separated into two groups (low-frequency, N=9, and high-frequency, N=12) with an average weight of 3.71 (±0.78) kg, were used in this study. Cats 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 dorsolumbar fascia, and an S-shaped stainless steel hook made of 1.5-mm-diameter rod was applied around the supraspinous ligament halfway between the dorsal processes of L4 and L5. The preparation was then positioned in a rigid stainless steel frame, and the lumbar spine was isolated by means of two external fixators that were applied to the L1 and L7 posterior process (feline has seven lumbar vertebrae). The external fixation was intended to limit the elicited flexion to the lumbar spine and prevent interaction of thoracic and sacral and/or pelvic structures but not to prevent any motion. A schematic of the setup is shown in Fig. 1 as also published in our previous reports [6,28].

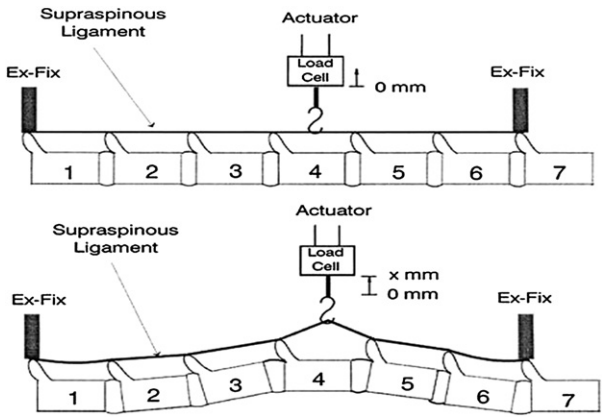

Fig. 1. (Top) A schematic diagram of the setup during rest and (Bottom) during peak load.

Instrumentation

The S-shaped stainless steel hook inserted around the L4–L5 supraspinous ligament was connected to the crosshead of the Bionix 858 Material Testing System (MTS, Minneapolis, MN, USA), in which a load cell and displacement sensor were 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. The load cell and displacement outputs of the Bionix 858 MTS were also sampled into the computer at 1,000 Hz. Under such loading condition, the lumbar spine underwent anterior flexion-extension while straining the lumbar supraspinous ligaments.

Protocol

A preload of 1 N was applied just before each single period of cyclic loading to produce a standard baseline across all preparations. A set of six 10-minute cyclic (sinusoidal) loading periods at 0.25 Hz (or one cycle every 4 seconds) and 40 N peak, each followed by 10-minutes rest, were applied for a cumulative cyclic loading period of 60 minutes. The following recovery phase consisted of 7 hours of rest at no load, during which single-test cycles of 40 N peak load at 0.25 Hz were applied. The single cycles were applied at 10, 30, and 60 minutes after termination of the cyclic loading period and then once every hour. Overall, nine test cycles were applied during the 7-hour recovery period. Fig. 2, Bottom trace, shows the schematic of the cyclic loading. Load and displacement were recorded throughout the protocol.

The high-frequency group was subjected to a cyclic load of 40 N peak but at 0.5 Hz instead of 0.25 Hz, whereas all other loading conditions remained the same as described above.

The baseline cyclic frequency of loading was selected to be 0.25 Hz based on previous measurements in normal humans performing deep flexion-extension at normal

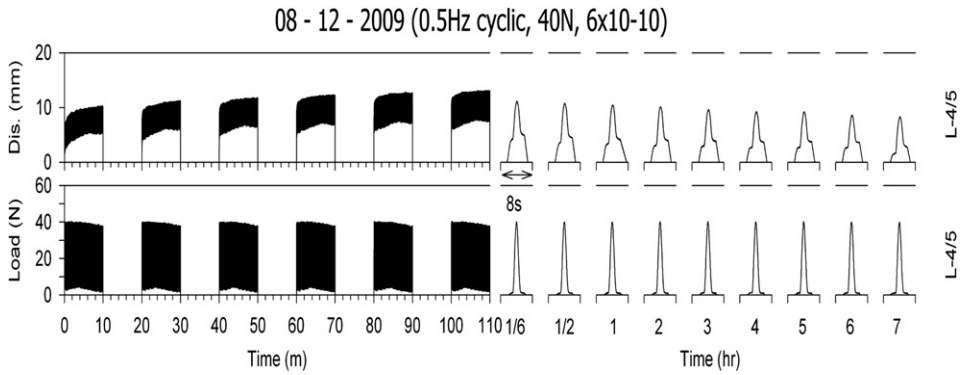

Fig. 2. A typical recording of cyclic load and the associated displacement during six 10-minute load/10-minute rest period and the following 7-hour rest period during which single cycle tests were taken to assess creep recovery.

comfortable pace. Furthermore, 40 N was selected as a moderate load based on our earlier studies [33].

At the end of the 7-hour recovery period, the supraspinous ligaments of the L3–L4, L4–L5, and L5–L6 were harvested from the specimen for cytokine analysis. For purposes of comparison, the supraspinous ligament of T10–T11 from each preparation was also removed for analysis as self-control because it was not subjected to the cyclic load or any associated motion. Comparison of lumbar ligaments to unstimulated thoracic ligament as self-control was scientifically advantageous. This, however, required confirmation that baseline/unstimulated cytokine levels in different ligaments of the same specimen were approximately the same. For validation of this assumption, a control group of animals was tested, and the validation was described in the appendix of a previous report [16].

Cytokines analysis

Ribonucleic acid extraction and preparation of cDNA

Ligaments were flash frozen in liquid nitrogen, stored at −80°C, then powdered in a laboratory ball mill (Mikro-Dismembrator S; Sartorious BBI Systems, Inc., Bethlehem, PA, USA). Ribonucleic acid was extracted and purified from the powdered ligaments using the RNeasy Lipid Tissue Mini Kit according to manufacturer's directions (QIAGEN, Valencia, CA, USA). The procedure included an on-column DNase step. The average concentration and purity of RNA extracted from all ligaments were 190 ng/μL and 2.16 (λ 260/280 nm), respectively. Complementary DNA (cDNA) was prepared from 1 μg of RNA isolated from each sample using the HC cDNA RT kit with RNase inhibitor (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR

Expression of gene targets was measured using real-time reverse transcription polymerase chain reaction. Primers and probes for interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNFα), and transforming growth factor (TGF)-β1 were designed with the assistance of the Primer Express sequence detection software (Applied Biosystems, Foster City, CA, USA). Cytokines are soluble proteins that respond to ligament injury by binding to their receptors and initiating a cellular response [34]. Interleukin-1 is a strong proinflammatory cytokine that works both directly and indirectly. Interleukin-1 induces B-cell differentiation and acute-phase proteins [35]. It also drives extracellular matrix destruction by increasing degradative enzymes, such as matrix metalloproteinases [36]. In addition, IL-1 stimulates fibroblasts to express and secrete other inflammatory cytokines including IL-6 [37,38]. The action of IL-6 is typically proinflammatory during the acute phase by inducing the production of acute-phase proteins (some distinct from those produced by IL-1 and TNFα) and can also promote B- and T-cell functions and stimulate the secretion of immunoglobins [38,39]. Interleukin-8 is considered a chemokine because it attracts neutrophils to the site of inflammation [40], for example, in inflammatory joint disease [41]. The action of TNFα is similar to that of IL-1 but is thought to be less potent in most tissues. The remaining cytokine measured in this study, TGFβ, is a multifunctional cytokine that acts in normal physiology and pathology. It may act in potentially contradictory ways depending on tissue and pathology [42]. In inflammation, TGFβ 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, USA) were prepared with 5′-label of 6-carboxy fluorescein and 3′-label of 6-carboxy-tetramethylrhodamine. A 50-μL reaction mix containing TaqMan Universal Master Mix, forward and reverse primers, probes, RNase-free water, and 1 μg of the template cDNA was amplified for each target using an ABI prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) with an initial melt at 95°C for 10 minutes followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute. Real-time data acquisition and analysis were performed using threshold cycle values, in which mRNA levels for each gene were normalized to the corresponding expression levels of glyceraldehyde-3-phosphate dehydrogenase. 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.

Statistical analysis

Cytokine expression levels (IL-1β, IL-6, IL-8, TGFβ, and TNFα) for L3–L4, L4–L5, and L5–L6 were divided by the cytokine expression levels in the T10–T11 reference ligament harvest from the same cat for normalization. Comparison between the different loading rates was accomplished using a two-way mixed model analysis of variance, where loading rate (0.25 Hz and 0.5 Hz) and vertebral level (L3–L4, L4–L5, and L5–L6) were fixed variables and specimen was a random variable. On significant interactions or main effects in both tests, post hoc analyses were performed using Student *t* tests to delineate statistically significant effects. All data were visually inspected for a normal distribution, and an appropriate data transformation was applied, if necessary. Before data transformation, the highest and lowest values from each loading rate×vertebral-level grouping were removed before any analyses were performed. Level of significance was set at 0.05.

Results

The mean displacement (±SD) and the mean-calculated creep from the specimen used in the low-frequency loading are shown in Fig. 3, Top. The creep developed by the end of the first 10-minute cyclic loading was 26.2%. It recovered to 18.4% at the end of the first 10-minute rest. The creep continued to increase and partially recovered in the

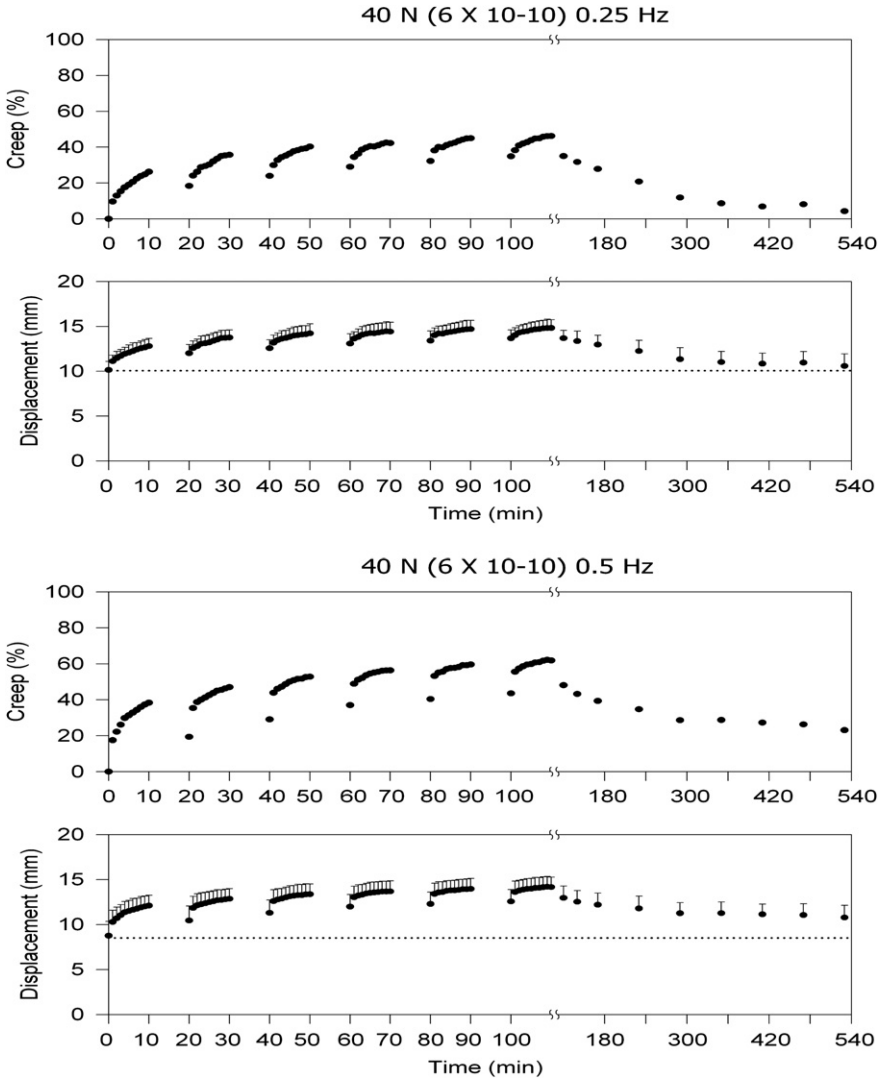

Fig. 3. The displacement and the associated creep developed in the lumbar spine during the cyclic loading and recovery during the following 7-hours rest. (Top) The creep behavior of the low-frequency group is shown and (Bottom) that of the high-frequency group.

following five loading/rest sessions, reaching a final value of 46.2% at the end of the six loading/rest sessions. During the 7-hour rest, the creep recovered exponentially [7], reaching a residual value of 4.3% by the end of the 7th hour.

Similarly, the mean displacement and creep from the specimen used in the high-frequency loading is shown in Fig. 3, Bottom. At the end of the first cyclic loading session, the creep was at 38.3%. It recovered to 19.4% at the end of the first rest period. The creep continued to increase and partially recovered in the following five loading/rest sessions, reaching 61.8% at the end of the six loading/rest sessions. The recovery during the 7-hour rest period was exponential [7], reaching a residual value of 23.1% by the end of the 7th hour.

The results of the statistical analysis are shown graphically in Fig. 4. Interleukin-6, IL-8, and TNFα expression were significantly increased in the group subjected to the 0.5-Hz loading frequency relative to the 0.25 Hz for the three vertebral levels. Significant increase in IL-1β expression was also present for L4–L5 and L5–L6 as well as for TGFβ expression for L5–L6 in the higher loading frequency group. Three of the cytokines (IL-1β, IL-8, and TGFβ) demonstrated significant interactions between loading rate and vertebral level. Post hoc analyses revealed that L4–L5 expression levels were greater than L3–L4 expression levels in each of these cytokines. Interleukin-6 and TNFα had significant main effects of loading frequency ($p=.020$ and $p=.037$, respectively), in which cytokine expression were significantly larger in the higher frequency

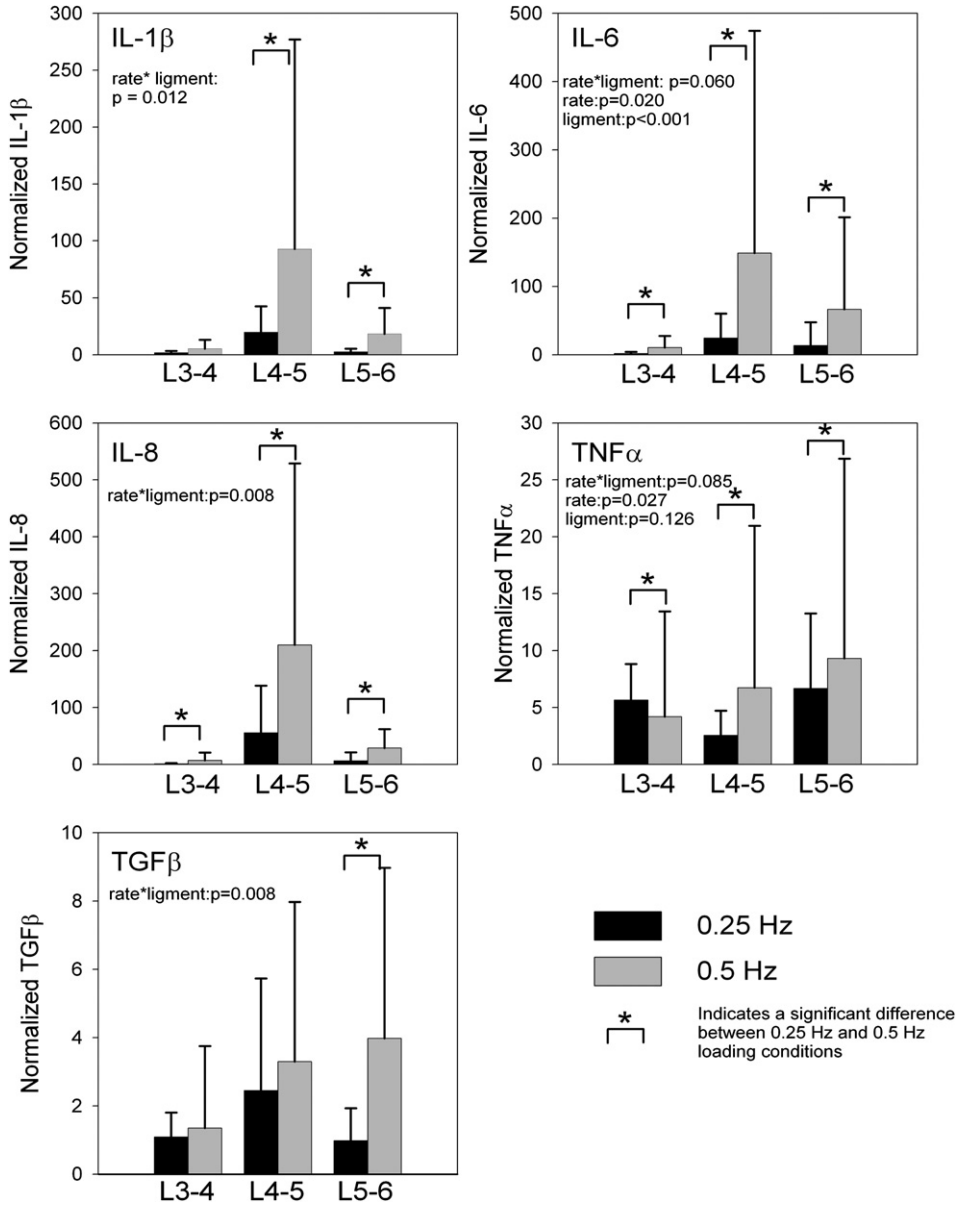

Fig. 4. Graphical presentation of the five cytokines' expression levels at low and high frequency and the associated statistical analysis.

Table
Statistical results of loading condition, vertebral level, and interaction

	p Value		
Cytokines	Interaction	Loading rate	Vertebral level
IL-1β	.012*	–	–
IL-6	.060	.020*	.001*
IL-8	.008*	–	–
TGFβ	.008*	–	–
TNFα	.085	.037*	.126

IL, interleukin; TNFα, tumor necrosis factor-α; TGFβ, transforming growth factor-β.

* Significant effect ($p<.05$).

group. Vertebral level also demonstrated a significant effect ($p=.001$) in IL-6 in which L4–L5 expression levels were larger then L3–L4 levels. Table provides the summary of the statistical analysis.

Discussion

The primary findings of this investigation reveal that prolonged cyclic loading of the lumbar supraspinous ligaments at a high frequency, within the physiological range, triggers significant increase in proinflammatory cytokines expression relative to that observed at a lower frequency. The new experimental data confirm and support epidemiological reports [2–5] identifying high-velocity movements in the occupational setting as a major risk factor for CTD. The new data also confirm basic science reports assessing the impact of higher flexion-extension frequency as a major triggering source of a neuromuscular disorder and substantial laxity observed in animal models [31,32] and development of high internal loads in various lumbar structures in humans [43–45].

Cyclic loading at a moderate frequency of 0.25 Hz consists of arriving to the peak load of 40 N within 2 seconds. This corresponds to an approximate loading rate of 20 N/second. Conversely, at a frequency of 0.5 Hz, the same peak load of 40 N is reached within 1 second. The approximate loading rate in this case is 40 N/second or twice that observed at the 0.25 Hz condition. Effectively, the loading rate of the supraspinous ligaments (and that of the other viscoelastic tissues, such as intraspinous ligaments, discs, facet capsule, and so on) was doubled and subjected the collagen fibers to a substantially higher tension development [29,30,45] and exposure to substantially more microruptures [11–13].

Indeed, the mean-residual creep of the high-frequency group (23.1%) was over 5× larger than that of the low-frequency group (4.3%), further suggesting that substantially larger microdamage was present in this condition. The large increase in subfailure microdamage of the collagen fibers within the supraspinous ligaments is most likely the source for the significant increase of proinflammatory cytokines in the ligaments subjected to high-frequency loading.

Several other issues should be considered. Because the frequency was doubled but the loading duration remained the same, the number of repetitions of flexion-extension doubled. Increase in the number of repetitions was shown by the epidemiology [2–4] and confirmed by experimental data [19] to be a risk factor for cumulative disorder. One could suspect, therefore, that the increased number of repetitions was an interactive factor in the significantly increased cytokines expression in the high-frequency group.

Conversely, a previous study compared the impact of low- and high-frequency (0.25 Hz and 0.5 Hz) flexion-extension on the development of the neuromuscular disorder associated with the inflammatory condition [32]. In that study, the load was kept light at 20 N, and the number of repetitions in the high-frequency (0.5 Hz) group was kept identical to that of the low-frequency (0.5 Hz) group. The cumulative duration of loading was, therefore, also half as much as that of the low-frequency group. Despite the much more favorable condition for the high-frequency group, a neuromuscular disorder developed, indicating that an inflammatory condition was present several hours after loading. The low-frequency group, however, did not exhibit a neuromuscular disorder.

In essence because the number of repetitions was identical and the loading duration was half as much, the only change between the two groups that could trigger the disorder in the high-frequency group was the doubling of the loading rate. It leads one to assert with greater confidence that high rates of loading of lumbar tissues is the most eminent factor in the development of proinflammatory conditions. High loads, high number of repetitions, and long loading duration seem to be secondary risk factors.

Our previous work demonstrated that loading conditions such as high loads and high loading frequency trigger excessive spasms in the lumbar multifidi and a delayed hyperexcitability of the same muscles several hours after loading [15]. This was also shown to be associated with ×100 increase in neutrophil density in the supraspinal ligaments. The elevated neutrophils level, spasms, and delayed hyperexcitability seem to be present only in loading conditions that could inflict microdamage in the viscoelastic tissues. Spasms during loading are known to represent occurring damage to tissues. It is also known that presence of inflammatory conditions in neurological tissue triggers hyperexcitability [46]. Overall, one can suggest that the high loading rate caused microdamage in the ligaments with the simultaneous associated spasms. The microdamage triggers, within several hours, significant increase in the presence of neutrophils and proinflammatory cytokines. That in turn triggers neuromuscular hyperexcitability, which was observed at the same time.

The development of proinflammatory condition in an animal repetitive injury model was described before for the upper extremity [47,48]. It is important to note, again, the large differences in the function of the lumbar ligaments. The physiologic strain of the anterior cruciate ligament,

for example, is 6% to 7% [49], whereas the physiologic strain of the lumbar supraspinous ligaments ranges from 24% to 42% [27,28] over 4× larger. Lumbar ligaments also seem to undergo reorientation during flexion [50]. Yet, despite the four- to five-fold larger physiologic strain range, the lumbar ligaments appear to be subjected to microdamage and proinflammatory degradation when exposed to prolonged repetitive activity of low and high loads [16,17], in general, and to high-frequency loading, in specific, as described in this report.

Although the data were derived from an animal model, its validity in humans is anticipated because the viscoelastic tissues are of identical molecular structure, have nearly the same physiologic strain range, and the anatomy and biomechanics are closely related in structure and function [51]. Long-ranging implications can be suggested; if workers with significant increase in proinflammatory cytokines in the lumbar tissues continue to engage in daily long hours of work requiring high speed flexion-extension, their condition which is consistent with an acute inflammation, can convert to a chronic inflammation with the associated permanent tissue degeneration and a neuromuscular disorder, for example, CTD.

Conclusions

It is concluded that the prolonged performance of lumbar flexion-extension at high velocity triggers significant increase in proinflammatory cytokines expression within the supraspinous ligaments, an acute inflammation, and overall decrease in the integrity of the viscoelastic tissues. This may have an important insight on the understanding of CTD.

Acknowledgments

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