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Inflammatory cytokine expression in the quadriceps of rats with posttraumatic knee stiffness: A preliminary study

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ABSTRACT

The primary purpose of this study was to investigate cytokine expression in the quadriceps of rats with posttraumatic knee stiffness (PTKS) and to determine the effect of exercise training on these cytokines at different follow-up time points. The PTKS rats were randomly assigned into two even groups. The treatment group received exercise training, while the control group received no treatment. Quadriceps specimens were harvested randomly from each group at 8, 12, 16, and 20 weeks. RT-qPCR and immunohistochemical analyses were used to assess the protein and mRNA expression levels of the cytokines IL-1, IL-2, TNF-α, COX-1, and COX-2. TNF-α immunostaining did not differ between the treated and control group tissues, whereas weak immunostaining was observed for all other cytokines in the specimens from the treatment group compared with those from the control group at approximately 12 and 20 weeks. The cytokine levels decreased at approximately 8 weeks in the treatment group, whereas these levels remained elevated or plateaued in the control group. These differences were statistically significant ($p < 0.05$). This study demonstrated that the expression of cytokines IL-1, IL-2, COX-1, and COX-2 increased in the quadriceps of rats with PTKS and that exercise training affected the observed profile trends of these cytokines.

1. Introduction

Posttraumatic joint stiffness is a common complication that occurs primarily after extremity injuries involving articular structures. The periarticular structures (arthrogenic) and the muscles (myogenic) are the factors that are responsible for joint contracture and stiffness. Most studies have indicated that arthrogenic factors, particularly the joint capsule, play an important role in developing joint stiffness. However, according to the co-authors' experience, the muscles around the joint are clinically critical to developing posttraumatic joint stiffness and to rehabilitative managing this stiffness [1].

Recent studies have demonstrated that pro-fibrotic cytokines play an important role in the pathogenesis of arthrofibrosis and posttraumatic joint stiffness [2]. However, most of these studies focused on cytokine expression in the capsule of the involved joint [3,4]. Few studies have examined the molecular alterations that occur within skeletal muscle (knee extensors) in this population. Other studies [5–8] have also revealed that chronic endurance exercise training reduces proinflammatory cytokine protein expression in rat skeletal muscle. Given the importance of the knee extensor muscles in terms of daily function and the role of proinflammatory cytokines in the pathogenesis of posttraumatic knee stiffness (PTKS) [1,2], investigating cytokine expression in knee extensor muscles may provide new insights into the molecular regulation of muscle function in patients with PTKS.

The primary goals of this study were to measure the protein and mRNA expression levels of interleukin 1 (IL-1), interleukin 2 (IL-2), tumour necrosis factor alpha (TNF-α), cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) in quadriceps harvested from an animal model of PTKS and to **preliminarily** evaluate the effects of exercise training on the expression of these cytokines at different follow-up time points.

2. Material and methods

2.1. Animal model

After obtaining approval from the institutional animal research committee of the China Rehabilitation Research Centre (CRRC, Beijing, China), forty skeletally mature Sprague–Dawley (SD) male

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rats (age: 9 weeks old, body weight: 300–400 g) were studied (Experimental Animal Centre, Academy of Military Medical Sciences, Beijing, China). After the animals were anaesthetised by intra-peritoneal injection of 10% chloral hydrate (0.5 ml/100 g), 2×2 mm^2 of cortical bone from the lateral femoral condyles was removed to create a cortical window. This unilateral knee joint was rigidly immobilised using femorotibial wire at full extension (0°) as described previously [9].

The animals were housed in $60 \times 40 \times 30$ cm plastic cages lined with chips, with 2 animals per cage, a 12-h light/12-h dark cycle, and rat chow and water available ad libitum. The room temperature and humidity were maintained at 18 °C and 50–55%, respectively. Knee stiffness on the experimental side developed as the animals were kept in their cages for 4 weeks post-surgery [10]. The femorotibial wire was removed in a second surgery at the end of 4 weeks of immobilisation under the same anaesthesia described above.

2.2. Study design and tissue samples

Before the surgery (0 weeks), four normal rats were sacrificed randomly, and the quadriceps were harvested to measure the baseline levels of cytokine protein and mRNA expression. Four rats with stiff knees after 4 weeks of immobilisation were sacrificed to obtain quadriceps for study. The other thirty-two rats were randomly assigned to two groups, treatment and control, with sixteen rats in each group. The treatment group was treated the day after the second surgery with exercise therapy, including chronic manual stretching (two times/day and 15 min/period, applied by an investigator within the endurance of the non-anaesthetised rat), six-channel rat treadmill exercise (Columbus Inc., USA, and a flat treadmill setting with a speed of 0.3 m/s, two times/day and 15 min/period). The control group did not receive any rehabilitative management. Four rats were selected randomly from each group and sacrificed to harvest the quadriceps specimens at 8, 12, 16, and 20 weeks after the first surgery. When the rats were ambulating freely before sacrifice, the knee joint range of motion (ROM) was compared visually between the two groups at all time points. The joint improvement level was recorded as slight ($\leqq$30° flexion) or obvious ($\geqq$90° flexion).

The muscle tissue immediately above the quadriceps tendon was harvested as a sample. The same sample was used for immunohistochemical and RT-qPCR analyses. Three areas were included for the analysis of staining density, and the highest integral optical density (IOD) value was recorded for analysis. All tissue samples underwent haematoxylin and eosin (H&E) staining, and the extent of muscle degeneration and fibrosis was observed using an optical microscope.

The assessment of cytokine protein and mRNA levels was conducted by the staff at the Medical Molecular Biology Laboratory of Peking Union Medical College Hospital, Peking, China. The laboratory staff performed the quantitative analysis and were blinded to the study design. The findings are expressed as numerical values.

2.3. RT-qPCR analysis

The muscle samples were stored at −80 °C in liquid nitrogen until further processing for RNA isolation to assess specific mRNA levels. RT-qPCR was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines [11].

Total RNA was extracted using an ultrapure RNA extraction kit (Cat# CW0581). Then, the obtained total RNA was reverse transcribed using a HiFi-MMLV cDNA kit (Cat# CW0744). Real Super Mixture with Rox (Cat# CW0767), DNase 1 (Cat# CW2090), and a fluorescence-based quantitative real-time PCR instrument (ADI7500) were used to perform RT-qPCR. All above-mentioned reagents and equipment used for this analysis were supplied by Perkin Elmer Inc., USA. The primer sequences used in the RT-qPCR analysis are shown in Table 1. The ΔΔCT method was used for the data analysis to determine fold changes in expression between the treated and control tissues [11].

Table 1
Details of primer pairs used.

Gene	Sequence of primer	Product size (base pairs)
IL-1	F: CAAAGAAGAAGATGGAAAAGCGGTT R: CTATGTCCCGACCATTGCTG	156
TNF	F: GGGCAGGTCTACTTTGGAGTCATTG R: GGGCTCTGAGGAGTAGACGATAAAG	128
COX-1	F: TCCTACATGGGATGACGAGC R: GGTTGCGATACTGGAACTGG	167
IL-2	F: GCACTGACGCTTGTCCTCCTTG R: ATGTTTCAATTCTGTGGCCTGCTT	195
COX-2	F: AACCACCTCTATCACTGGCATCCG R: CAAAGTTCCTACCCCCACCAATCC	178
RAT ACTIN	F: GGAGATTACTGCCCTGGCTCCTA R: GACTCATCGTACTCCTGCTTGCTG	150

R: reverse, F: forward.

2.4. Immunohistochemistry

The specimens were fixed in 10% formalin, embedded in paraffin and cut into 4-μm sections using a microtome. The antibody reagents used to treat the sections included IL-1 (sc9983) and IL-2 (sc7896), which were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and TNF-α (AB1793), COX-1 (AB109025), and COX-2 (AB15191), which were supplied by Abcam Ltd. (Cambridge, UK). The IOD values, which were determined using Image Pro-Plus (IPP) software, were used to evaluate the cytokine protein concentrations.

2.5. Statistical analysis

Statistical analysis was performed using ANOVA and SPSS statistics software. The Student–Newman–Keuls (SNK)-q test was used to analyse the expression levels of cytokines between the study and control tissues at different follow-up time points. The level of significance was proposed as $p < 0.05$. The data are presented as the mean ± standard deviation (m ± SD).

3. Results

The knee joint ROM on the experimental side improved slightly at 8 and 12 weeks in the treatment group but was obvious at 16 and 20 weeks, whereas knee stiffness remained unaltered in the control group. Measurable levels of IL-1, IL-2, TNF-α, COX-1, COX-2 protein and mRNA expression were detected in all of the samples.

3.1. Immunohistochemistry

Figs. 1 and 2 present the immunostaining results of IL-1 and COX-1, which are two of the five cytokines analysed in the quadriceps in this study. Weak immunostaining was observed in the treatment group tissues compared with that in the control group tissues at approximately 12, 16 and 20 weeks. IL-2 and COX-2 immunostaining demonstrated the same results as IL-1 and COX-1 immunostaining, in contrast to TNF-α immunostaining, which did not differ between the control and treatment group tissues.