COMBINED REHABILITATIVE EXERCISE AND CELECOXIB TREATMENT RELIEVES INFLAMMATION AND SMOOTHS QUADRICEPS MUSCLE CONTRACTION AFTER TRAUMATIC KNEE INJURY

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ABSTRACT

Background*: Traumatic knee injury is common and highly debilitating. Its signs and symptoms usually persist long enough to produce muscle atrophy and, sometimes, even permanent contractile dysfunction.*

Aim*: Our goal was to evaluate the effect of combined rehabilitative exercise and celecoxib treatment on the inflammatory reaction in spastic rat quadriceps after traumatic knee injury.*

Methods*: Rats were randomly assigned to one of four subgroups: a control group, exercise group, celecoxib treatment group, and combined rehabilitative exercise and celecoxib treatment. From each group, quadriceps muscles were collected four, eight, 12, 16, and 20 weeks after traumatic knee injury. In addition to range of motion in each rat, expression of IL-1, IL-2, TNF-a, COX-1, and COX-2 was evaluated at each time point using real-time polymerase chain reaction and immunohistochemistry.*

Results*: Our results showed that, consistent with an apparent decrease in range of motion after knee injury, expression of the cytokines IL-1, IL-2, COX-1, and COX-2 significantly increased in the spastic rat quadriceps, which was at least partially reversed using combined rehabilitative exercise and celecoxib treatment, resulting in reduced expression of those molecules and improved behavioral outcomes.*

Conclusion*: This study clearly shows that combined rehabilitative exercise and celecoxib treatment relieves acute and chronic inflammation in spastic rat quadriceps after traumatic knee injury.*

Keywords: *rehabilitative exercise, celecoxib, drug therapy, inflammatory reaction, quadriceps spasticity, traumatic knee injury.*

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Introduction

About 5% of the population aged 35 to 54 years has clinical sign and symptoms of knee osteoarthritis[1]. Of these, most are diagnosed after traumatic knee injury. Notably, joint injury, including knee injury, is the highest risk factor for the development of knee osteoarthritis in these young adults[1-4]. Risk factors of this injury, including obesity, aging, crystal deposition, muscle weakness and neuropathy, instability and malalignment, are usually caused by falling or colliding during sports or car accidents. They are also major knee-related factors contributing to suddenly increasing knee loads and, eventually, traumatic knee injury[5].

The knee joint is a complicated structure exquisitely composed of parts interacting with each other to provide proper alignment, stability, and motion. Of all these parts, muscles such as quadriceps are directly associated with bones by the cable-like tendons in the knee joint, which work together to extend the leg. Accordingly, traumatic knee injury inevitably results in an expanded spectrum response of inflammatory reactions in the tendons and muscles next to the knee joint through capillary circulation and/or lymphatic capillary circulation[1, 5]. Although the precise mechanism underlying inflammatory myopathies remains unknown, in the past decades, a number of studies of idiopathic inflammatory myopathies (eg, pathogenesis of certain autoimmune

diseases) have brought many novel insights into the causes of inflammatory muscle responses.

For instance, in dermatomyositis, complements and membranolytic attack complex deposit on the endothelial cells, resulting in focal losses of endomysial capillaries (perifascicular atrophy)[6-8], whereas the remaining capillaries have to dilate to compensate (endofascicular hypoperfusion)[6-8].

Moreover, membranolytic attack complex activates the release of proinflammatory cytokines (eg, interleukin (IL)-1 and IL-2), which are secreted by macrophages and lymphocytes, respectively, as well as tumor necrosis factor (TNF) α. Subsequently, these cytokines aid activated lymphocytes (eg, CD4+ T cells, B cells, dendritic cells) to migrate toward the perimysial and endomysial spaces through upregulation of type I interferon–inducible protein signaling[9] followed by interferon-β/MHC class I mediated autoamplification[10]. Likewise, in inclusion-body myositis or polymyositis, CD8+ cytotoxic T cells containing perforin granules invade nonnecrotic muscle fibers, aberrantly expressing MHC class I, which cause myonecrosis on release[11-13]. In brief, it is critical to suppress inflammatory reactions to relieve signs and symptoms of knee injury, particularly for the inflammatory muscle responses.

Celecoxib, the first-discovered cyclo-oxygenase 2 (COX 2)-selective inhibitor available in clinical practice[14] was approved worldwide to relieve the signs and symptoms of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and other inflammatory diseases[15, 16]. It also is highly effective as a nonselective nonsteroidal anti-inflammatory drug used to relieve acute and chronic musculoskeletal symptoms after traumatic knee injury[17]. Celecoxib is about 30 times more potent at suppressing COX-2 than COX-1.

Apart from drug treatment, patients with myositis caused by traumatic knee injury or other diseases generally receive rehabilitative exercise right away because reports have suggested that all aspects of rehabilitative exercise should be included until clinical symptoms are sufficiently resolved during long-term therapy, except for avoiding exercise in the acute phase[18].

Furthermore, our previous study demonstrated that the expression of both IL-1/2 and COX-1/2 increased in spastic rat quadriceps after traumatic knee injury but were at least partially reversed by rehabilitative exercise[19]. Nevertheless, the clinical effect of combined drug treatment and rehabilitative exercise on inflammatory reactions after knee joint injury was unknown.

Material and methods

Animals and experimental design

All animal experiments conducted in this study followed protocols approved by the Animal Care and Use Committee of the Academy of Military Medical Sciences and Institutional Animal Research Committee at the China Rehabilitation Research Centre (Beijing). Nine-week-old male Sprague-Dawley (SD) rats (n = 96) at starting weights of 300 to 400 g were obtained from animal facilities at the Academy of Military Medical Sciences. The rats were housed in 60 × 40 × 30 cm plastic cages lined with chips. Pairs were kept in each cage, where they were fed ad libitum and exposed to a 12-hour shift of light and dark at 18° C and 50% to 55% relative humidity. The rats were equally and randomly assigned to one of four groups (ie, control group, rehabilitative exercise group, celecoxib treatment group, and rehabilitative exercise combined with celecoxib treatment group).

Experimental model and tissue collection

Four rats from each group were randomly chosen from each group before traumatic knee injury (ie, cortical fractures of the lateral femoral condyle). The animal model of traumatic knee injury was produced as previously reported[19, 20]. Briefly, after anesthesia with intra-peritoneal injection of ketamine 80 mg/kg + xylazine 10 mg/kg , 2 × 2 mm2 of cortical bone from the lateral femoralcondyles was removed to create a cortical window. This unilateral knee joint was rigidly immobilized using femorotibial wire at full extension (0o).

Subsequently, rats from the rehabilitative exercise group received rehabilitative exercise, including 15-minute manual stretching, which was applied by an investigator twice per day below the maximum tolerated limit of the awakened rats, in addition to 15-minutes of treadmill exercise twice daily using a six-lane rat treadmill (Columbus Instruments, Inc., OH, USA) at a speed of about 0.3 m/s. Comparatively, rats from the celecoxib treatment group received celecoxib treatment (20 mg/kg body weight per day; Pfizer, NY, USA) at regular times using gastric gavage and did not receive rehabilitative exercise. However, rats from the combined exercise and celecoxib group received rehabilitative exercise after celecoxib treatment.

Accordingly, rats from the control group did not receive either therapy.

Before they were sacrificed, the rats were allowed to ambulate freely in order to determine their knee joint range of motion (ROM) by visual detection zero, four, eight, 12, 16, and 20 weeks after injury. The knee joint recovery scale was estimated as slight, defined as less than a 30-degree flexion contracture in the ROM, or obvious, defined as a more than 90-degree flexion contracture in ROM. After ROM detection, six rats from each group were randomly chosen, anesthetized via peritoneal injection of ketamine 80 mg/kg + xylazine 10 mg/kg, and sacrificed via cervical dislocation at each time point to collect their quadriceps muscles, which were subsequently stored at -80 oC for further analysis of their expression of both mRNA and protein. The researchers ensured that the rats were in cardiac arrest and had dilated pupils after they were sacrificed.

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) and the primer sequences used in this study were in accordance with the methods as we previously reported[21]. Briefly, the quadriceps muscles were processed for total RNA isolation using an RNA Extraction Kit (CW0581; CWBio, Beijing, China). The HiFi-MMLV cDNA Kit (CW0744; CWBio) was applied for first-strand cDNA synthesis, followed by RT-qPCR with Real Super Mixture and Rox (CW0767; CWBio) and DNase 1 (CW2090; CWBio) using a fluorescence-based RT-qPCR instrument (ADI7500; Thermo Fisher Scientific, MA, USA)[21].

Immunohistochemistry

Immunohistochemistry was conducted in accordance with the methods we previously reported[21]. After tissue preparation with 10% formalin fixation and paraffin embedding, sections (4-μm thickness) of the samples were obtained onto polylysine-coated slides using a microtome followed by a 20-minute treatment with hyaluronidase (100740; Seikogaku, Inc., Tokyo, Japan). The slides were incubated by the primary antibodies, including IL-1 (sc9983; Santa Cruz, Inc., CA, USA), IL-2 (sc7896; Santa Cruz, Inc.), TNF-a (AB1793; Abcam Ltd., Cambridge, UK), COX-1 (AB109025; Abcam Ltd.), and COX-2 (AB15191; Abcam Ltd.) and detected using corresponding secondary antibodies, which were evaluated by using integral optical density (IOD) with Image Pro-Plus software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were statistically analyzed using SPSS V. 22 (IBM, NY, USA). The data were expressed as means ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with a least significant difference (LSD) test for post hoc analysis. The significance level was set at $P < 0.05$.

Results

Effect of combined rehabilitative exercise and celecoxib treatment on ROM in spastic rat quadriceps after traumatic knee injury

Before being sacrificed, we examined each rat's ROM corresponding to their spastic quadriceps during ambulation. The results showed the effect of rehabilitative exercise and/or drug treatment on ROM in spastic quadriceps at each time point after traumatic knee injury. We found that, although there was is significant difference in ROM in rats that received rehabilitative exercise or celecoxib treatment within two months after injury, rehabilitative exercise obviously expanded the ROM at 12 weeks ($^{**}P < 0.01$ vs. control group, without celecoxib treatment; $^{***}P < 0.001$ vs. control group, with celecoxib treatment), 16 ($^{***}P < 0.001$ vs. control group, with or without celecoxib treatment), and 20 weeks after injury ($P < 0.001$ vs. control group; with or without celecoxib treatment) (Table 1).

Notably, celecoxib treatment only vs. rehabilitative exercise combined with celecoxib treatment caused a significant decrease in ROM in rats at 12 weeks ($^{\Delta\Delta}P < 0.01$ vs. exercise + celecoxib group), 16 weeks ($^{\Delta\Delta\Delta}P < 0.001$ vs. exercise + celecoxib group), and 20 weeks ($^{\Delta\Delta\Delta}P < 0.001$ vs. exercise + celecoxib group) after injury (Table 1). Likewise, celecoxib treatment only reduced ROM at 12 weeks ($^{\#}P < 0.05$ vs. exercise group), 16 weeks ($^{\#\#\#}P < 0.001$ vs. exercise group), and 20 weeks ($^{\#\#\#}P < 0.001$ vs. exercise group) after injury compared with that of rats in the exercise group (Table 1).

Taken together, these results indicate that celecoxib treatment only did not significantly improve ROM in spastic quadriceps after traumatic knee injury. Notably, combined celecoxib treatment and rehabilitative exercise resulted in the most improvement 12 weeks after injury of all the groups.

Group	Values (M ± SD) at different time point (weeks)					
	0	4	8	12	16	20
Control	0.55 ± 0.25	1.22 ± 0.90	11.50 ± 1.29	30.75 ± 3.30	61.50 ± 2.65	80.50 ± 4.20
Exercise	0.63 ± 0.27	1.57 ± 0.80	10.75 ± 1.50	37.25 ± 2.22**	80.00 ± 3.74***	115.75 ± 4.35***
Exercise + Celecoxib	0.88 ± 0.14	1.38 ± 0.73	11.00 ± 0.82	41.00 ± 3.37***	80.75 ± 4.65***	121.50 ± 3.11***
Celecoxib	0.74 ± 0.17	1.01 ± 0.25	11.86 ± 0.50	31.54 ± 2.02$^{\#\#\#}\Delta\Delta\Delta$	63.52 ± 2.49$^{\#\#\#}\Delta\Delta\Delta$	80.65 ± 2.71$^{\#\#\#}\Delta\Delta\Delta$

Table 1: Effect of rehabilitative exercise and celecoxib treatment on range of motion in spastic quadriceps muscle following traumatic knee injury.

$^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $\#\#\#P < 0.001$ vs. exercise; $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs. exercise + celecoxib

Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of IL-1, IL-2, and TNFα in spastic quadriceps after traumatic knee injury

Proinflammatory cytokines, including IL-1, IL-12, TNFα, and others, are types of signaling molecules excreted from T cells and macrophages to promote inflammation and are involved in mediating the innate immune response. After traumatic knee injury, excessive chronic release of IL-1, IL-12, and TNFα contributes to myositis-like symptoms and severe spasticity.

To determine the underlying mechanism in attenuation of muscle spasticity with combined rehabilitative exercise and celecoxib treatment, we examined the expression of those proinflammatory cytokines using RT-qPCR and immunohistochemistry (Figures 1 and 2; Tables 2 and 3). Interestingly, compared with other groups, rats from the celecoxib group showed the most decrease and the minimum expression of IL-1/IL-2 in both protein and mRNA level during acute-phase inflammatory response (i.e., four weeks ($^{***}P < 0.001$ vs. control group; $^{\#\#\#}P < 0.001$ vs. exercise group; $\Delta\Delta\Delta P < 0.001$ vs. exercise + celecoxib group) and eight weeks ($^{***}P < 0.001$ vs. control group; $^{\#\#\#}P < 0.001$ vs. exercise group; $\Delta\Delta\Delta P < 0.001$ vs. exercise + celecoxib group) after injury (Figures 1 and 2; Tables 2 and 3).

Three months after injury, however, expression of IL-1/IL-2 in rats from R + D group took over the bottom position and showed a significant difference compared with that of the control group ($^{***}P < 0.001$ vs. control group) or exercise group ($^{\#\#\#}P < 0.001$ vs. exercise group), at least in the protein level. Moreover, during the chronic-phase inflammatory response (ie, 16 and 20 weeks after injury, rehabilitative exercise became the major factor negatively regulating the expression of IL-1/IL-2 compared with the control group, which was reflected by a significant decrease in that of rats with ($^{***}P < 0.001$ vs. control group) or without ($^{***}P < 0.001$ vs. control group) celecoxib treatment. Comparatively, expression of TNFα showed no significant difference among groups and increased slightly within three months after injury (as show Figure 3; Tables 2 and 3).

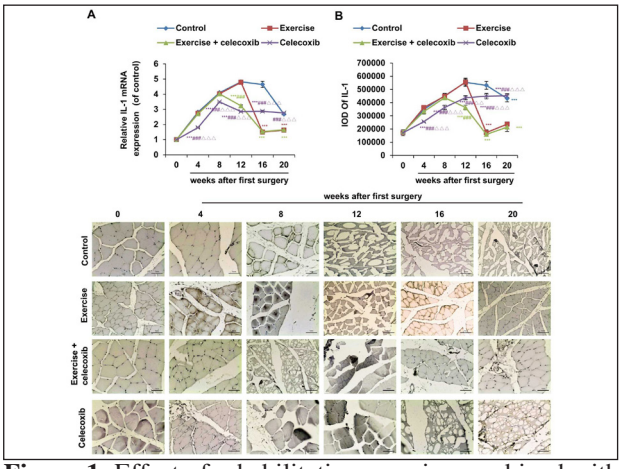

Figure 1: Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression level of IL-1 in spastic quadriceps after traumatic knee injury. (**A**) IL-1 mRNA expression was determined by real-time polymerase chain reaction. ß-actin was used as an inner control. (B) IL-1 protein expression was determined using immunohistochemistry. Scale bar: 1 mm. Data are shown as mean ± standard deviation (n = 4 at each time point and group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. exercise; $\Delta\Delta\Delta P < 0.001$ vs. exercise and celecoxib.

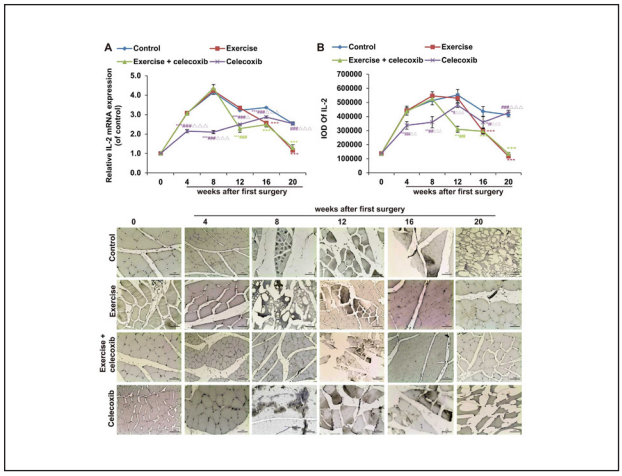

Figure 2: Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of IL-2 in spastic quadriceps after traumatic knee injury. (**A**) IL-2 mRNA expression level was determined by real-time polymerase chain reaction. ß-actin was used as an inner control. (**B**) IL-2 protein expression was determined using immunohistochemistry. Scale bar: 1 mm. Data are shown as mean ± standard deviation (n = 4 at each time point and group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. exercise; $\Delta\Delta\Delta P < 0.001$ vs. exercise and celecoxib.

Cytokines Group		Values (X ± SD) at different time point (weeks)					
		0	4	8	12	16	20
IL-1	Control	171987.8 ± 14283.6	348850.0 ± 15111.2	451798.7 ± 16469.9	554537.3 ± 31147.5	531132.6 ± 29962.6	431132.6 ± 25892.6
	Exercise	171987.8 ± 22598.3	363096.8 ± 10256.3	449013.7 ± 20819.3	555708.7 ± 22901.9	173067.4 ± 21031.2***	236977.2 ± 15460.1***
	Exercise + Celecoxib	176459.1 ± 10233.2	333096.5 ± 19102.6	436977.2 ± 15460.1	363581.5 ± 17502.1$^{***\#\#\#}$	159653.8 ± 10496.3***	214477.2 ± 32889.8***
	Celecoxib	171734.1 ± 4385.8	255878.6 ± 4209.3$^{***\#\#\#}\Delta\Delta\Delta$	363581.5 ± 17502.6$^{***\#\#\#}\Delta\Delta\Delta$	436977.1 ± 15460.1$^{***\#\#\#}\Delta\Delta$	449013.7 ± 20819.3$^{***\#\#\#}\Delta\Delta\Delta$	451798.1 ± 16469.9$^{\#\#\#}\Delta\Delta\Delta$
IL-2	Control	137710.1 ± 8695.3	441789.2 ± 31362.5	513965.8 ± 31285.6	552721.2 ± 39529.2	436125.9 ± 33685.3	412596.3 ± 15663.6
	Exercise	137709.8 ± 8694.7	441789.2 ± 31362.5	546623.8 ± 28953.5	530518.7 ± 30209.9	295568.3 ± 20615.9***	117824.2 ± 13140.9***
	Exercise + Celecoxib	136087.2 ± 6938.5	436885.6 ± 27314.3	525289.1 ± 18952.2.1	308895.7 ± 20564.5$^{***\#\#\#}$	295483.6 ± 16889.2***	136087.2 ± 6938.5***
	Celecoxib	137434.6 ± 8603.1	336885.6 ± 27314.3$^{***\#\#\#}\Delta\Delta\Delta$	359284.2 ± 40462.4$^{***\#\#\#}\Delta\Delta\Delta$	479756.1 ± 15442.8$^{***\#}\Delta\Delta\Delta$	359284.2 ± 40462.4$^{***\#\#}\Delta\Delta\Delta$	426885.6 ± 17386.4$^{\#\#\#}\Delta\Delta\Delta$
TNF-α	Control	568665.5 ± 12753.6	634057.2 ± 27282.8	421595.1 ± 28553.6	388526.1 ± 44369.0	336856.5 ± 19562.3	409562.1 ± 20593.5
	Exercise	564165.5 ± 17078.2	634057.6 ± 27282.6	406983.6 ± 25986.5	365483.3 ± 25996.2	365594.5 ± 16589.2	386691.4 ± 26983.6
	Exercise + Celecoxib	564165.6 ± 17078.2	638827.5 ± 23198.8	449549.7 ± 35481.7	421269.5 ± 33025.6	353085.5 ± 1812.5	355632.2 ± 33569.2
	Celecoxib	464557.1 ± 14930.7	435908.6 ± 19211.3	553012.5 ± 17399.6	371550.9 ± 15563.2	352984.6 ± 16048.9	270485.2 ± 12700.1
COX-1	Control	265615.5 ± 42967.8	337876.2 ± 27686.5	659201.5 ± 28693.7	500364.2 ± 27020.1***	580147.3 ± 30659.8	686510.8 ± 17456.8
	Exercise	258115.5 ± 42491.8	337876.6 ± 27686.7	634462.0 ± 34136.9	392256.3 ± 30598.6	356281.1 ± 22396.2***	162539.2 ± 20125.6***
	Exercise + Celecoxib	258115.3 ± 42491.5	337876.3 ± 27686.9	523485.5 ± 27553.3$^{***\#\#}$	308869.2 ± 31258.2$^{***\#\#\#}$	215633.3 ± 24751.3$^{***\#\#\#}$	132845.2 ± 15639.2$^{***\#}$
	Celecoxib	247085.3 ± 26613.6	337785.5 ± 27152.6	420019.4 ± 48085.6$^{***\#\#\#}\Delta\Delta$	450151.2 ± 21258.4$_{***\#}\Delta\Delta\Delta$	521626.0 ± 31642.5$^{*\#\#\#}\Delta\Delta\Delta$	675730.3 ± 18572.4$^{\#\#\#}\Delta\Delta\Delta$
COX-2	Control	103326.2 ± 9587.9	267453 ± 24935.8	508508.4 ± 30875.2	524367.7 ± 22895.6	553515.7 ± 19488.7	521333.6 ± 26733.8
	Exercise	106576.2 ± 9217.8	272453.5 ± 21852.3	505232.9 ± 28514.3	305625.3 ± 26514.2***	309696.3 ± 19335.9***	126369.3 ± 20532.1***
	Exercise + Celecoxib	106576.3 ± 12660.9	272453.6 ± 29462.6	335892.2 ± 45882.5$^{***\#\#\#}$	261289.5 ± 15482.3$^{***\#}$	169103.8 ± 14750.4$^{***\#\#\#}$	150002.3 ± 18596.2***
	Celecoxib	103404.2 ± 7955.4	258923.6 ± 15947.7	405994.3 ± 17184.2$^{***\#\#}\Delta\Delta$	415991.5 ± 17184.9 $^{***\#\#\#}\Delta\Delta\Delta$	456776.3 ± 18301.2$^{***\#\#\#}\Delta\Delta\Delta$	516481.5 ± 12528.3$^{\#\#\#}\Delta\Delta\Delta$

Table 2: The IOD detection of each molecule at different time points.

$^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. exercise; $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs. exercise + celecoxib

In brief, except for TNFα, expression of proinflammatory cytokines, including IL-1 and IL-12, are most efficiently suppressed by celecoxib treatment only during the acute-phase inflammatory response. However, during the chronic-phase inflammatory response, rehabilitative exercise plays a predominant role in reducing their expression. Moreover, combined rehabilitative exercise and celecoxib treatment is most effective in the in-between phase after injury.

Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of COX-1 and COX-2 in spastic quadriceps after traumatic knee injury

Pharmaceutical inhibition of COX using many nonsteroidal anti-inflammatory drugs (eg, celecoxib, aspirin, ibuprofen) usually provides sufficient relief from the inflammatory reactions of many symptoms. Traditionally, celecoxib is considered a COX-2-selective inhibitor, being 30 times more potent at suppressing COX-2 than COX-1.

To examine its selective attenuation of muscle spasticity during rehabilitative exercise combined with celecoxib treatment, we examined expression of the COXs using RT-qPCR and immunohistochemistry (Figures 4 and 5; Tables 2 and 3). Interestingly, during the late acute phase (ie, eight weeks after injury), expression of COX-1 of rats in the celecoxib group had a minimal increase in both mRNA and protein ($^{***}P < 0.001$ vs. control group; $^{\#\#\#}P < 0.001$ vs. exercise group; $\Delta\Delta P < 0.01$ vs. exercise + celecoxib group). However, expression of COX-2 in rats in the exercise and celecoxib group took the bottom position in mRNA and protein levels ($^{***}P < 0.001$ vs. control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. exercise group). Furthermore, consistent with the reduced expression trend for proinflammatory

	Group	Values (X ± SD) at different time point (weeks)					
	0	4	8	12	16	20	
IL-1	Control	1.000 ± 0.000	2.812 ± 0.068	4.097 ± 0.060	4.817 ± 0.073	4.645 ± 0.206	2.676 ± 0.035
	Exercise	1.000 ± 0.000	2.737 ± 0.029	4.027 ± 0.051	4.785 ± 0.085	1.507 ± 0.051***	1.625 ± 0.055***
	Exercise + Celecoxib	1.000 ± 0.000	2.705 ± 0.031	4.022 ± 0.038	3.232 ± 0.062***###	1.537 ± 0.023***	1.653 ± 0.057***
	Celecoxib	1.000 ± 0.000	1.800 ± 0.039***###ΔΔΔ	3.491 ± 0.053***###ΔΔΔ	2.855 ± 0.035***###ΔΔΔ	2.875 ± 0.061***###ΔΔΔ	2.762 ± 0.053###ΔΔΔ
IL-2	Control	1.000 ± 0.000	3.073 ± 0.055	4.153 ± 0.115	3.225 ± 0.038	3.361 ± 0.036	2.544 ± 0.086
	Exercise	1.000 ± 0.000	3.073 ± 0.055	4.228 ± 0.029	3.336 ± 0.035	2.565 ± 0.043***	1.159 ± 0.009***
	Exercise + Celecoxib	1.000 ± 0.000	3.056 ± 0.041	4.356 ± 0.189	2.277 ± 0.185***###	2.483 ± 0.121***	1.298 ± 0.152***
	Celecoxib	1.000 ± 0.000	2.147 ± 0.074***###ΔΔΔ	2.101 ± 0.081***###ΔΔΔ	2.495 ± 0.033***###Δ	2.873 ± 0.057***###ΔΔΔ	2.537 ± 0.047###ΔΔΔ
TNF-α	Control	1.000 ± 0.000	2.402 ± 0.029	3.849 ± 0.035	2.508 ± 0.021	1.658 ± 0.009	0.603 ± 0.011
	Exercise	1.000 ± 0.000	2.409 ± 0.036	3.822 ± 0.018	2.541 ± 0.018	1.637 ± 0.017	0.601 ± 0.005
	Exercise + Celecoxib	1.000 ± 0.000	2.353 ± 0.099	3.856 ± 0.115	2.545 ± 0.010	1.564 ± 0.099	0.604 ± 0.004
	Celecoxib	1.000 ± 0.000	2.339 ± 0.085	3.814 ± 0.079	2.526 ± 0.021	1.639 ± 0.039	0.599 ± 0.007
COX-1	Control	1.000 ± 0.000	3.265 ± 0.046	4.540 ± 0.101	4.501 ± 0.030	4.247 ± 0.023	4.641 ± 0.101
	Exercise	1.000 ± 0.000	3.263 ± 0.041	4.557 ± 0.026	2.549 ± 0.051***	2.692 ± 0.011***	1.983 ± 0.078***
	Exercise + Celecoxib	1.000 ± 0.000	3.248 ± 0.047	3.495 ± 0.323***###	2.395 ± 0.052###	2.112 ± 0.058***###	2.033 ± 0.044***
	Celecoxib	1.000 ± 0.000	3.225 ± 0.017	3.041 ± 0.050***###ΔΔ	3.504 ± 0.365***###ΔΔΔ	3.568 ± 0.094***###ΔΔΔ	4.601 ± 0.057###ΔΔΔ
COX-2	Control	1.000 ± 0.000	2.150 ± 0.070	4.049 ± 0.033	4.452 ± 0.026	4.310 ± 0.048	4.218 ± 0.095
	Exercise	1.000 ± 0.000	2.151 ± 0.069	4.030 ± 0.019	4.353 ± 0.487	2.826 ± 0.035***	2.163 ± 0.040***
	Exercise +	1.000 ± 0.000	2.193 ± 0.097	3.526 ± 0.088***###	2.168 ± 0.100***###	1.554 ± 0.069***###	2.136 ± 0.019***
	Celecoxib	1.000±0.000	2.181±0.044	3.026±0.069 *** ###ΔΔΔ	3.226±0.078 *** ###ΔΔΔ	4.048±0.033 *** ###ΔΔΔ	4.278±0.048###ΔΔΔ

Table 3: The mRNA expression level of each molecule at different time points.
***P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ###P < 0.001 vs. exercise; ΔΔP < 0.01, ΔΔΔP <0.001 vs. exercise + celecoxib*

cytokines, expression of COX-2 (***P < 0.001 vs. control group; #P < 0.05), and COX-1 (***P < 0.001 vs. control group; ###P < 0.001 vs. exercise group) in rats treated with combined treatment use took the bottom position for mRNA and protein levels 12 weeks after injury. Likewise, combined use continued to play a predominant role in suppressing COX-1 (***P < 0.001 vs. control group; ###P < 0.001 vs. exercise group) and COX-2 (***P < 0.001 vs. control group; ###P < 0.001 vs. exercise group) of mRNA and protein, even in the early chronic phase (ie, 16 weeks after injury). Nevertheless, expression of both COX-1 (***P < 0.001 vs. control group; #P < 0.05 vs. exercise group) and COX-2 (***P < 0.001 vs. control group) showed a minimal amount and a maximum decrease of mRNA and protein in rats treated with rehabilitative exercise with or without celecoxib treatment during the late chronic phase (ie, 20 weeks after injury).

In summary, expression of COXs, in particular COX-1, is most effectively inhibited by celecoxib treatment only during the late acute-phase inflammatory response. However, during the late chronic phase, rehabilitative exercise plays a predominant role in reducing their expression. Moreover, combined use of both rehabilitative exercise and celecoxib treatment is most effective during the in-between and early chronic phase after injury.

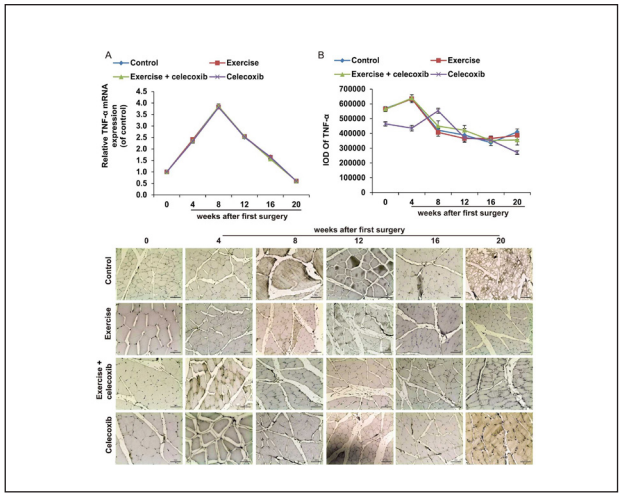

Figure 3: Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of TNFα in spastic quadriceps after traumatic knee injury. (**A**) TNF-α mRNA expression was determined by real-time polymerase chain reaction. ß-actin was used as an inner control. (**B**) TNF-α protein expression was determined using immunohistochemistry. Scale bar: 1 mm. Data are shown as mean ± standard deviation (n = 4 at each time point and group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. exercise; $\Delta\Delta\Delta P < 0.001$ vs. exercise and celecoxib.

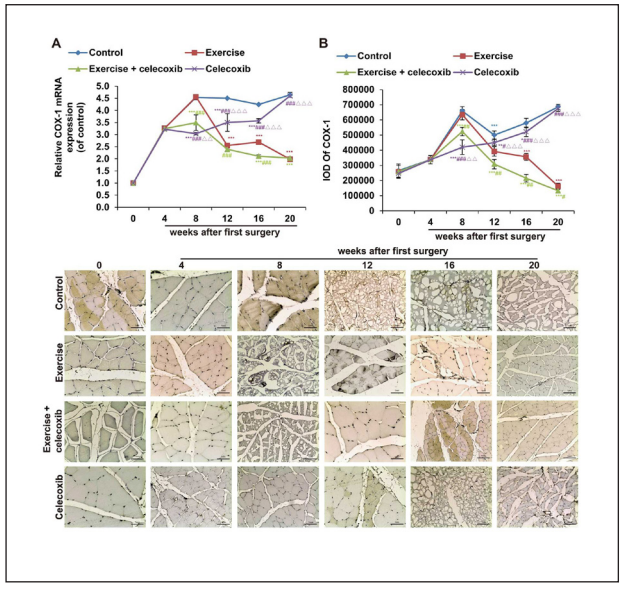

Figure 4: Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of COX-1 in spastic quadriceps after traumatic knee injury. (**A**) COX-1 mRNA expression was determined by real-time polymerase chain reaction. ß-actin was used as an inner control. (**B**) COX-1 protein expression was determined using immunohistochemistry. Scale bar: 1 mm. Data are shown as mean ± standard deviation (n = 4 at each time point and group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. exercise; $\Delta\Delta\Delta P < 0.001$ vs. exercise and celecoxib.

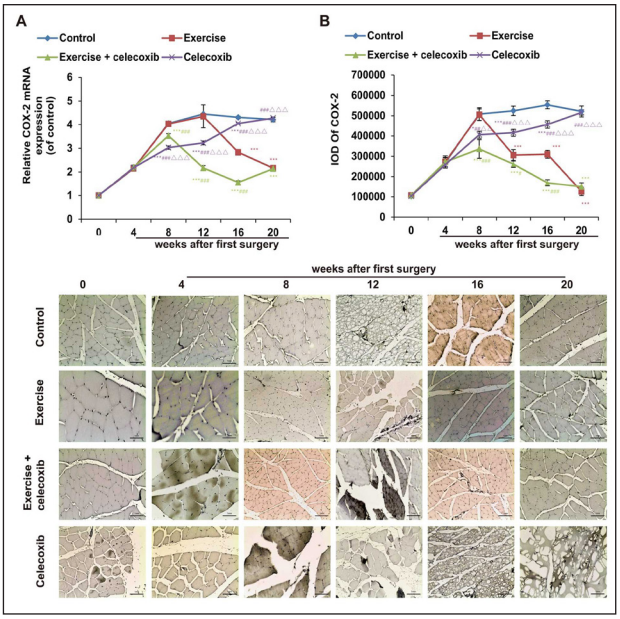

Figure 5: Effect of rehabilitative exercise combined with celecoxib treatment on mRNA and protein expression of COX-2 in spastic quadriceps after traumatic knee injury. (**A**) COX-2 mRNA expression was determined by real-time polymerase chain reaction. ß-actin was used as an inner control. (**B**) COX-2 protein expression was determined using immunohistochemistry. Scale bar: 1 mm. Data are shown as mean ± standard deviation (n = 4 at each time point and group). **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ###P < 0.001 vs. exercise; ΔΔΔP < 0.001 vs. exercise and celecoxib.

Discussion

Our study demonstrated that the expression of cytokines IL-1, IL-2, COX-1, and COX-2 increased in the spastic quadriceps of rats after traumatic knee injury. Rehabilitative exercise and celecoxib treatment affected the cytokine expression profiles of IL-1, IL-2, COX-1, and COX-2 but not TNF-a. Moreover, celecoxib treatment took the lead in decreasing expression of proinflammatory molecules during the acute-phase inflammatory response (ie, four and eight weeks after injury). However, rehabilitative exercise took over the top position in reduction of inflammation response during the chronic phase (ie, 16 and 20 weeks after injury), particularly for the late chronic-phase inflammatory response (ie, 20 weeks after injury). Therefore, combined rehabilitative exercise and celecoxib treatment became most predominant in the in-between phase (ie, 12 weeks after injury).

Celecoxib has been shown to affect clinical dose proportionality of the therapeutic dosage range. Celecoxib is generally metabolized by the P450 2C9 isoenzyme, or hepatic cytochrome, into

inactive molecules, resulting in the production of urine and fecal excretion[14, 15]. Steady-state plasma concentrations are normally detected within five days, although peak plasma concentrations are reached as soon as three hours after controlled oral drug delivery[14, 15].

In our study, rats in the celecoxib or exercise plus celecoxib group received controlled oral drug delivery on a daily basis at regular time intervals. Thus, it is reasonable that celecoxib efficiently suppressed the induction of proinflammatory cytokines (eg, IL-1/IL-2) and other inflammatory molecules (eg, COX-1/COX-2) as early as four weeks after injury.

Although nonsteroidal anti-inflammatory drugs (eg, aspirin, celecoxib) can alleviate the inflammatory response, drug resistance has been reported in cancers, diseases, and injuries (22-25). For instance, about 18% of women claimed no response to dysmenorrhea when using these drugs and had to pursue less-studied strategies[24]. Notably, rs20417 is a critical type of single nucleotide polymorphism (SNP) in the promoter sequence of the COX-2 gene, which is highly correlated with aspirin resistance[26]. Although combined rehabilitative exercise and celecoxib treatment was most predominant 12 weeks after injury, results indicated that celecoxib drug resistance developed during the chronic-phase inflammatory response, and particularly in the late chronic phase, revealing that the potentially updated expression profile during the chronic phase may play an essential role in late-onset celecoxib drug resistance.

COX-1 and COX-2 share a 63% similarity of identical amino acid sequences and have a highly homologous catalytic binding site[27]. Celecoxib, a COX-2 selective inhibitor of many diseases (14), has been shown to have an almost identical effect on expression of COX-1 and COX-2 in spastic rat quadriceps after traumatic knee injury. The results strongly hint of a potentially novel cross-talk between the COX-1- and COX-2-mediated signaling pathway in such circumstances. Still, further investigation is needed to determine how to link them to attenuate the inflammatory response, smoothing spastic quadriceps after traumatic knee injury, at least within three months after injury.

Conclusion

In conclusion, our study showed that the expression of cytokines IL-1, IL-2, COX-1, and COX-2 increased in the quadriceps of rats with PTKS at first mention.(PTKS). Exercise and drug therapy affected the cytokine expression profiles of IL-1, IL-2, COX-1, and COX-2 but not TNF-a. Higher levels of cytokine expression in the control group were consistent with the extent of muscle degeneration and fibrosis. These findings suggest that elevated levels of inflammatory cytokines in the quadriceps might contribute to the pathophysiology of PTKS. But animal model cannot be fully adopted in human conditions.If you want to use experimental results in humans, you need more scientific experiments for research.

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Abbreviations:
Interleukin (IL); tumor necrosis factor (TNF); Sprague-Dawley (SD), range of motion (ROM), quantitative real-time PCR (RT-qPCR), integral optical density (IOD), standard deviation (SD), analysis of variance (ANOVA), least significant difference (LSD), single nucleotide polymorphisms (SNP), International Council for Laboratory Animal Science (ICLAS).

Ethics approval:
All animal experiments in this study followed protocols approved by the Animal Care and Use Committee of the Academy of Military Medical Sciences and Institutional Animal Research Committee of the China Rehabilitation Research Centre (Beijing, China).

Authors' contributions
Fei Wang conceived and coordinated the study; designed, performed, and analyzed the experiments; and wrote the paper. Fei Wang, Sihai liu, Zhigang Cui, and Anqing Wang carried out the data collection and data analysis and revised the paper. Fei Wang and Jiangjun Li had full access to all data and take responsibility for its integrity and the accuracy of the data analysis. All authors were involved in drafting the article or revising it critically for important intellectual content. All authors reviewed the results and approved the final version of the manuscript.

Availability of data and material
The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

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