Effects of Cyclic Stretching Exercise on Long-Lasting Hyperalgesia, Joint Contracture, and Muscle Injury Following Cast Immobilization in Rats

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Short title: Effect of stretching exercise on immobilized rat hindlimbs

Summary

The effects of exercise on mechanical hyperalgesia, joint contracture, and muscle injury resulting from immobilization are not completely understood. This study aimed to investigate the effects of cyclic stretching on these parameters in a rat model of chronic post-cast pain (CPCP). Seventeen 8-week-old Wistar rats were randomly assigned to (1) control group, (2) immobilization (CPCP) group, or (3) immobilization and stretching exercise (CPCP+STR) group. In the CPCP and CPCP+STR groups, both hindlimbs of each rat were immobilized in full plantar flexion with a plaster cast for a 4-week period. In the CPCP+STR group, cyclic stretching exercise was performed 6 days/week for 2 weeks, beginning immediately after cast removal prior to reloading. Although mechanical hyperalgesia in the plantar skin and calf muscle, ankle joint contracture, and gastrocnemius muscle injury were observed in both immobilized groups, these changes were significantly less severe in the CPCP+STR group than in the CPCP group. These results clearly demonstrate the beneficial effect of cyclic stretching exercises on widespread mechanical hyperalgesia, joint contracture, and muscle injury in a rat model of CPCP.

Key words: Stretching exercise, Hyperalgesia, Muscle damage, Immobilization

45 **Introduction**

46 Chronic periods of reduced physical activity can occur following traumatic injury,
47 with prolonged immobilization, and as a part of aging. The primary effects of muscle disuse
48 in such situations include progressive skeletal muscle atrophy (Honda *et al*. 2015), loss of
49 muscle extensibility (Honda *et al*. 2018), and joint contracture (Inoue *et al*. 2007, Morimoto *et*
50 *al*. 2013). Studies have confirmed that 4 weeks of hindlimb cast immobilization causes disuse
51 muscle atrophy in rats (Okita *et al.* 2009), with decreased capillary-to-myofiber ratios in the
52 hindlimb muscles after 2 weeks (Kataoka *et al.* 2014) and 4 weeks (Matsumoto *et al.* 2014) of
53 immobilization. Other studies have shown that cast immobilization induces muscle fibrosis,
54 which contributes to limb contracture (Honda *et al.* 2015; Maezawa *et al.* 2017, Yoshimura *et*
55 *al.* 2017). A 4-week period of hindlimb cast immobilization was shown to increase the
56 vulnerability of rats to muscle damage at reloading because of alterations in mobility and
57 movement (Inoue *et al*. 2009).

58 In addition to physical and functional changes, recent studies in healthy human
59 subjects and animal models have found that prolonged immobilization induces pain
60 hypersensitivity (Terkelsen *et al*. 2008, Nakano *et al*. 2012, Ohmichi *et al*. 2012, Morimoto *et*
61 *al*. 2013, Sekino *et al*. 2014, Hamaue *et al*. 2015, Nakagawa *et al*. 2018) and may contribute
62 to the development of complex regional pain syndrome (Allen *et al*. 1999). A study of healthy
63 rats with 2-week cast immobilization of one hindlimb found long-lasting skin and muscle

64 hyperalgesia in the immobilized and contralateral limbs (chronic post-cast pain; CPCP)
65 (Ohmichi *et al*. 2012).

66 Immobilization-induced hyperalgesia and joint contracture affect the recovery of
67 muscle functionality after immobilization, limit activities of daily living, and increase
68 healthcare costs. Various therapeutic strategies for reducing CPCP and joint contracture,
69 including treadmill exercises (Morimoto *et al*. 2013), vibration exercises (Hamaue *et al*.
70 2015), and static stretching (Morimoto *et al.* 2013), have been evaluated in animal models.
71 However, the effects of stretching exercise on post-immobilization pain and joint contracture
72 remain unclear. Some studies have found that stretching reduces joint contracture (Kaneguchi
73 *et al.* 2019), whereas others have not found a clinically relevant effect (Harvey *et al.* 2017).
74 Continuous passive motion on a stretching machine was shown to decrease markers of
75 inflammation and mitigate hyperalgesia in a rat model of arthritis (Nakabayashi *et al.* 2016).
76 Similarly, stretching exercises reduced inflammation and improved pain in rats with
77 subcutaneous inflammation induced by carrageenan (Corey *et al*. 2012). One recent animal
78 study reported that static stretching decreased pain and increased joint range of motion
79 (ROM) in a rat model of CPCP (Morimoto *et al*. 2013). However, to our knowledge, no
80 studies have evaluated the effect of cyclic stretching initiated immediately after cast removal
81 on post-immobilization muscle pain in a rat model of CPCP. The hypothesis of this study was
82 that cyclic stretching exercises initiated immediately after cast removal would decrease long-

lasting post-immobilization mechanical hyperalgesia in rats. We also evaluated the effect of cyclic stretching on post-immobilization joint contracture and muscle damage.

Methods

Animals

All experiments were approved by the Ethics Committee for Animal Experimentation at the Nagoya University School of Health Science. This study was performed in compliance with the ethical guidelines of the International Association for the Study of Pain and the European Guidelines on Laboratory Animal Care.

Seventeen 8-week-old male Wistar rats were purchased from Japan SLC (Hamamatsu, Japan) and housed under a 12-h light/dark cycle with free access to food and water. The rats were randomly divided into the following three groups: CPCP without cyclic stretching exercises (CPCP, n=6), CPCP with cyclic stretching exercises (CPCP+STR, n=6), and age-matched naïve controls (CON, n=5; **Figure 1A**).

Immobilization and reloading

CPCP was generated through 4 weeks of hindlimb cast immobilization (Nakagawa *et al*. 2018). Rats in the CPCP and CPCP+STR groups were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg). The bilateral hindlimbs were encased for 4 weeks in plaster casts (Alcare, Tokyo, Japan) in full plantar flexion from just above the knee to the distal foot. Casts were replaced every 2 to 3 days to prevent loosening and hindpaw edema. When the immobilized rats were anesthetized, the age-matched controls (CON group) were also anesthetized to avoid possible confounding. Pentobarbital sodium was the only medication

105 administered during the study period. After the 4-week immobilization period, casts were
106 removed and animals were allowed to ambulate freely in their cages.
107
108 ***Stretching exercises***
109 Stretching exercises were modified from Inoue *et al*. (2009). Rats in the CPCP+STR
110 group were anesthetized as above and the bilateral gastrocnemius muscles were stretched with
111 a custom-built apparatus (**Figure 1B**). The hindlimb was stabilized with hip and knee
112 extended by taping the foot to the platform, which was connected to a movable board attached
113 to a shaft. The amplitude and frequency of cyclical stretches were controlled with a stepping
114 motor. Stretching exercises were performed at a frequency of once every 4 s with a range of
115 40° from maximum dorsiflexion, as measured with a goniometer. The cyclical stretching was
116 performed for 30 min/day, 6 days/week, beginning immediately after cast removal (prior to
117 reloading) and continuing for 2 weeks (12 sessions total).
118
119 ***Behavior tests***
120 Behavior tests to assess mechanical sensitivity in the calf muscle and hindpaw skin
121 were performed before cast immobilization (baseline), prior to reloading immediately after
122 cast removal (Day 0), and on Days 1, 3, 5, 7, 10, and 14 after cast removal. The tests were
123 performed prior to stretching on each testing day. During these tests, rats were wrapped
124 individually in a cloth restrainer because ankle joint contracture prevented those in the

125 immobilized groups from walking on their hindlimbs. As shown in **Figures 1C** and **D**, the
126 restrainer allowed the animal to dangle safely with the legs positioned to be free and under no
127 loading, as described by Nakano *et al*. (2012).

128 A Randall–Selitto analgesiometer (Ugo Basile, Comerio, Italy) equipped with a
129 probe with a 2.6-mm tip diameter was used to measure the withdrawal threshold of the right
130 gastrocnemius muscle (**Figure 1C**). Use of a large-diameter probe enabled measurement of
131 the withdrawal threshold of deep tissue (Nasu *et al*. 2010). The nociceptive threshold was
132 defined as the force that induced a withdrawal response to an increasing pressure stimulus
133 from 0 to 2,450 mN. Measurements were repeated seven times at 2- to 3-min intervals; the
134 mean value in each session was taken as the withdrawal threshold.

135 The glabrous skin of the right hindpaw was probed six times with 2- and 7-g von
136 Frey filaments (VFFs; North Coast Medical, Morgan Hill, CA, USA) at 10-s intervals (**Figure**
137 **1D**). Lifting or pulling back the paw was counted as a paw withdrawal response. The 2- and
138 7-g filaments were used to ascertain mechanical allodynia and mechanical hyperalgesia,
139 respectively (Peleshok and Ribeiro-da-Silva 2011). This procedure was performed prior to the
140 Randall–Selitto test on each testing day.

141

142 ***Joint contracture***

143 Dorsiflexion ROM of the bilateral ankle joints was measured with a goniometer
144 (Inoue *et al*. 2007). Following the pain behavior tests, the rat was anesthetized and laid on its

145 side with the knee flexed to 90°. The ankle was passively dorsiflexed maximally and the angle
146 formed by the intersection of the line connecting the fifth metatarsal with the malleolus
147 lateralis and that connecting the malleolus lateralis with the center of the knee joint was
148 measured (0°–180°).
149
150 ***Histological analysis***
151 At the end of the experiment, the right gastrocnemius muscle of each animal was
152 excised under anesthesia with intraperitoneal pentobarbital sodium (50 mg/kg). The muscles
153 were embedded in an optimal cutting temperature compound (TissueTek®; Sakura Finetek,
154 Tokyo, Japan), quickly frozen by immersion in isopentane precooled in liquid nitrogen, and
155 processed for sectioning on a cryostat (CM1510-11; Leica, Wetzlar, Germany). Serial
156 transverse sections (7 μm) were cut from the muscle mid-belly and stained with
157 hematoxylin−eosin to assess muscle injury. Digital images of the stained sections were
158 acquired with an optical microscope (BZ-9000; Keyence, Osaka, Japan) at ×400
159 magnification (**Figure 1E and F**). Five image files were selected with a random number
160 table. Injured muscle fibers were defined as those displaying infiltration by more than two
161 nucleated inflammatory cells (**Figure 1E**) (Koh *et al*. 2003). Central nuclei were defined as
162 those located more than one nuclear diameter from the fiber border; myofibers with central
163 nuclei were termed centrally nucleated fibers (**Figure 1F**) (Zschüntzsch *et al*. 2016). A total of
164 10,000 muscle fibers contained in five images (image area, 1.5 × 1.2 mm) were analyzed with

Image J software (National Institutes of Health, Bethesda, MD, USA). The number of infiltrated muscle fibers and the number of centrally nucleated fibers per 10,000 fibers were used as indices of muscle injury.

Statistical analysis

Sigma Plot 13 (Systat Software, San Jose, CA, USA) was used for analyses. Because some dependent variables were not normally distributed according to Shapiro–Wilk testing, non-parametric tests were applied to all variables. The Friedman test was applied to compare differences in outcome measures between timepoints within each group. When a significant difference was found, a Dunnett's post-hoc test was performed to identify a significant difference from the baseline value. Differences between groups were analyzed with the Kruskal–Wallis test followed by a Dunn–Bonferroni post-hoc test for all pairwise multiple comparisons. P values <0.05 were considered significant. Graphs plot mean ± standard error of the mean (SEM), unless noted otherwise.

Results

Withdrawal thresholds of gastrocnemius muscle

Withdrawal thresholds immediately after cast removal in the CPCP and CPCP+STR groups were more than 20% lower than baseline values (from 216 to 165 g in CPCP group and from 217 to 158 g in the CPCP+STR group). These threshold values were significantly lower than that of the CON group (P=0.035 vs. CPCP and P=0.013 vs. CPCP+STR; **Figure 2**). The threshold reduction in the CPCP group was maintained over the 14-day study period and this threshold was always significantly lower than that of the CON group (P=0.002 on Days 1, 3, and 10; P<0.001 on Day 5; P=0.004 on Days 7 and 14). Conversely, the threshold reduction observed in the CPCP+STR group gradually recovered. By Day 1 after cast removal, there was no significant difference in threshold level between the CPCP+STR and CON groups. The threshold value of the CPCP+STR group was significantly higher than that of the CPCP group at 14 days after cast removal (P=0.036).

Paw-withdrawal responses

The number of paw-withdrawal responses elicited with 2-g VFFs is presented in **Figure 3A**. The number of responses after cast removal did not significantly differ from the number at baseline in any group at any point during the experimental period. However, the number of responses in the CPCP group was significantly higher than that in the CON group on Day 5 after cast removal (P<0.044).

199 The number of paw-withdrawal responses elicited with a 7-g VFF is presented in
200 **Figure 3B**. The number of responses in the CPCP group was significantly higher on Days 5
201 (P<0.001) and 7 (P=0.030) after cast removal compared with the number at baseline and was
202 higher than the number in the CON group on Days 1 (P=0.008), 5 (P=0.001), 7 (P=0.002), 10
203 (P=0.002), and 14 (P=0.006) after cast removal. The number of responses in the CPCP+STR
204 group was slightly but significantly increased on Day 5 after cast removal compared with
205 baseline (P=0.018); however, this value was not significantly different than that in the CON
206 group.
207
208 ***Range of motion of ankle dorsiflexion***
209 The ROM of bilateral ankle dorsiflexion is presented in **Figures 4A** and **B**. The
210 ROM in both hindlimbs immediately after cast removal was significantly lower than at
211 baseline in the CPCP and CPCP+STR groups (P<0.001 in both hindlimbs in each group). The
212 ROM gradually recovered over the study period. The ROM in the CPCP group was
213 significantly lower than that in the CON group over the 14-day period (right: P=0.037 on Day
214 0, P=0.003 on Day 1, P=0.004 on Day 3, P<0.001 on Days 5, 7, 10, and 14; left: P=0.018 on
215 Day 0, P=0.006 on Day 1, P<0.001 on Days 3, 5, 7, 10, and 14). Conversely, the ROM in the
216 CPCP+STR group did not significantly differ from than in the CON group on Day 3 or later.
217
218 ***Histological observations***

219 The gastrocnemius muscles of age-matched non-immobilized control rats (CON
220 group) displayed few myofibers with inflammatory infiltration or central nuclei. Conversely,
221 cellular infiltration and central nuclei were evident in the immobilized gastrocnemius muscles
222 (CPCP group) at 14 days following cast removal.
223 To assess the effects of stretching exercises on the number of fibers with
224 inflammatory infiltration and central nuclei, we evaluated the number of myofibers with these
225 findings per 10,000 fibers in each group. As shown in **Figure 5A** and **B**, the number of fibers
226 with infiltration and the number with central nuclei were both significantly higher in the
227 CPCP group than in the CON group (both P=0.004). Conversely, the number of fibers with
228 infiltration and the number with central nuclei in the CPCP+STR group did not significantly
229 differ from numbers in the CON group.

Discussion

Limb immobilization can cause prolonged joint contracture, muscle injury, and hyperalgesia, which can affect quality of life and increase healthcare costs. The present study revealed that cyclic stretching after hindlimb cast immobilization alleviated hyperalgesia, improved ROM, and limited muscle injury in a rat model of CPCP.

In this study, we used withdrawal responses to evaluate CPCP. Both immobilization groups (CPCP and CPCP+STR) had significantly lower pain thresholds on Day 0 after cast removal than at baseline, which confirms post-immobilization hyperalgesia in our model. However, the group treated with cyclic stretching had rapid amelioration of CPCP, with levels not significantly different from those in the control group by Day 1 after cast removal. Conversely, the CPCP group that was not treated with cyclic stretching had persistently low pain thresholds throughout the 2-week study period. These results are consistent with those of Morimoto *et al.* (2013), who reported that stretching ameliorated long-lasting hyperalgesia, joint limitation, and muscle atrophy induced by cast immobilization in rats. However, our study differed from that of Morimoto in the following respects. First, rats in the present study had a 4-week period of bilateral immobilization from just above the knee to the distal paw, whereas the previous study applied 2 weeks of unilateral immobilization from the trunk to the mid-hindpaw. Second, the present study used cyclic stretching applied six times/week for 2 weeks whereas the previous study used static stretching applied three times/week for 2 weeks. In a preliminary unpublished study, we compared the effects of static versus cyclic stretching

on muscle atrophy (fiber cross-sectional area) and injury (necrotic fiber number) after immobilization (Supplementary Methods section and Supplementary Tables 1 and 2). We found that cyclic stretching was superior to static stretching in ameliorating these conditions. Finally, stretching in the present study was initiated on the day of cast removal, before reloading, whereas stretching was initiated on Day 3 after cast removal in the study of Morimoto *et al*. The very early application of passive stretching resulted in significant amelioration in CPCP within 1 day of cast removal in the present study.

Joint contracture occurs during immobilization because of structural alterations, including muscle fibrosis and joint capsule changes (Wong *et al.* 2015). Studies have reported conflicting evidence regarding the efficacy of stretching in the treatment of immobilization-induced joint contracture. Several studies in animal models have found that stretching significantly improves joint ROM after immobilization (Inoue *et al.* 2007, Morimoto *et al.* 2013). However, a recent systematic review of 18 studies found that stretching did not have clinically important effects on joint contracture caused by various etiologies (Harvey *et al.* 2017). The present results support the efficacy of cyclic stretching in increasing the ROM of joints with immobilization-induced contracture.

In the present study, we used the presence of central nuclei and inflammatory cells within myofibers as markers of muscle injury. We found higher numbers of infiltrated and centrally nucleated muscle fibers in the gastrocnemius muscles of rats who underwent a 4-week immobilization period than in control rats. Central nuclei are a sign of muscle repair and

are seen in various types of muscular dystrophy and after muscle injury (Folker and Baylies 2013). The calf muscles of CPCP rats show disuse atrophy (Inoue *et al*. 2007); reloading of muscles with disuse atrophy induces inflammatory changes (Frenette *et al*. 2002). Therefore, the muscle injury in the present study may have resulted from reloading of the atrophic calf muscle. We found that early implementation of cyclic stretching significantly attenuated immobilization-induced muscle injury. This finding is consistent with that of Inoue *et al.* (2009), who demonstrated that stretching exercises performed soon after cast removal in rats decreased muscle injury (assessed based on inflammatory infiltration and heat shock proteins) in the cast-immobilized hindlimb. Similarly, Gomes *et al*. (2007) demonstrated that stretching exercises protected rat gastrocnemius muscles from atrophy and muscle damage during disuse. Although the relationship between muscle injury and CPCP is not clear, our finding that stretching decreased muscle injury and alleviated pain suggests that muscle damage may play a role in the development of CPCP. Further studies are needed to clarify this relationship.

This study has several limitations. First, it did not investigate the epidermis, spinal plasticity, or oxidative stress. Second, muscle injury was assessed with two parameters on hematoxylin–eosin staining only. Evaluation of additional histopathologic and systemic parameters could enhance our understanding of the effects of stretching on CPCP. Further detailed investigation of these aspects will be useful to elucidate the mechanisms by which stretching exercises decrease the pain associated with cast immobilization. In addition,

Schwann cells and muscle spindles could also be potential targets for exploring the mechanisms.

In conclusion, early implementation of cyclic stretching exercises ameliorated cutaneous and muscular mechanical hyperalgesia, joint contracture, and immobilization-induced muscle injury in a rat model of CPCP. Stretching exercises may decrease long-lasting hyperalgesia in patients undergoing rehabilitation following cast immobilization.

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Disclosures

The authors have no conflicts of interests to declare.

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Figure captions

Figure 1. Schematic diagram and photos of experimental protocol and representative photomicrographs of muscle tissue (hematoxylin–eosin staining).

(**A**) Treatment groups and treatment schedule. Rats were divided into three groups: age-matched naïve controls (CON, n=5), chronic post-cast pain (CPCP) without cyclic stretching exercise (CPCP, n=6), and CPCP with cyclic stretching exercise (CPCP+STR, n=6). (**B**) Photograph showing application of stretching exercise. Stretching was performed cyclically in the direction of plantar and dorsiflexion (in the range of 40° from maximum dorsiflexion) using a stretch apparatus at a frequency of once every 4 s for 30 min/day, 6 days/week. (**C**) Mechanical sensitivity of the gastrocnemius muscle was evaluated with a Randall–Selitto apparatus. (**D**) Mechanical sensitivity of the glabrous skin of the hindpaw was evaluated with von Frey filaments. (**E, F**) Representative photomicrographs of infiltrated muscle fiber (**E**) and centrally nucleated muscle fiber (**F**). Black and white arrows indicate infiltrated fibers and centrally nucleated fibers, respectively. Scale bar, 100 μm.

Figure 2. Time course of changes in withdrawal thresholds of gastrocnemius muscle.

Horizontal axis indicates measurement time points. Data are presented as mean ± SEM (n=5 or 6). *P<0.05 relative to associated baseline values; $^{\#}P$<0.05 relative to CON group; $^{\dagger}P$<0.05 relative to CPCP group.

Figure 3. Time course of changes in number of paw-withdrawal responses.

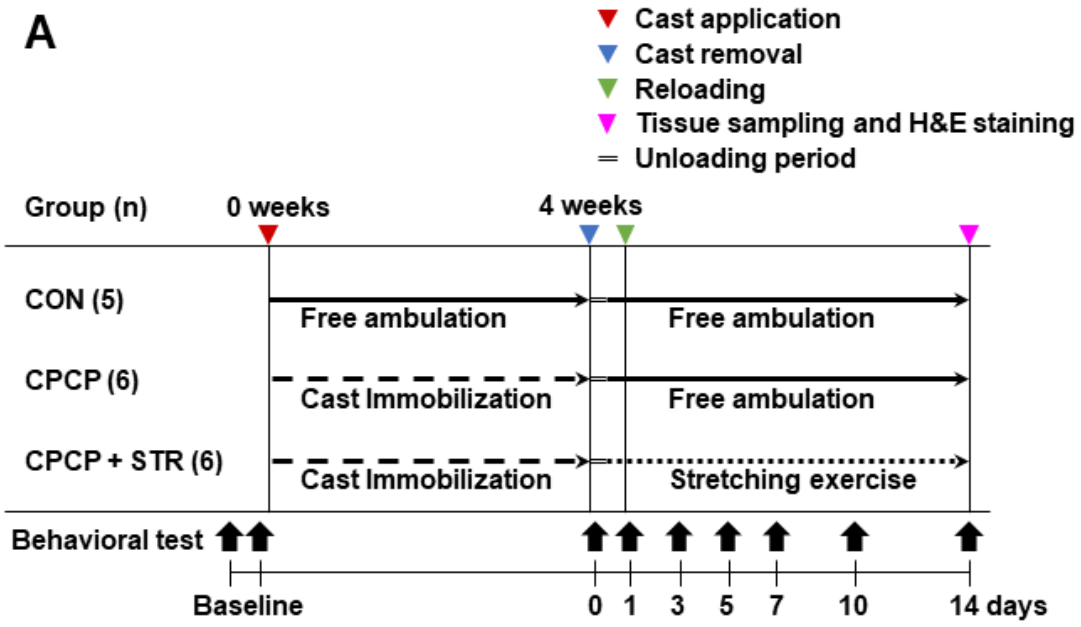
(**A**) Measurement of mechanical allodynia with 2-g von Frey filament (VFF). (**B**) Measurement of mechanical hyperalgesia with 7-g VFF. Horizontal axis indicates measurement time points. Data are presented as mean ± SEM (n=5 or 6). **P*<0.05 relative to baseline values; $^{\#}P$<0.05 relative to CON group.

Figure 4. Time course of changes in range of motion (ROM) of ankle dorsiflexion.

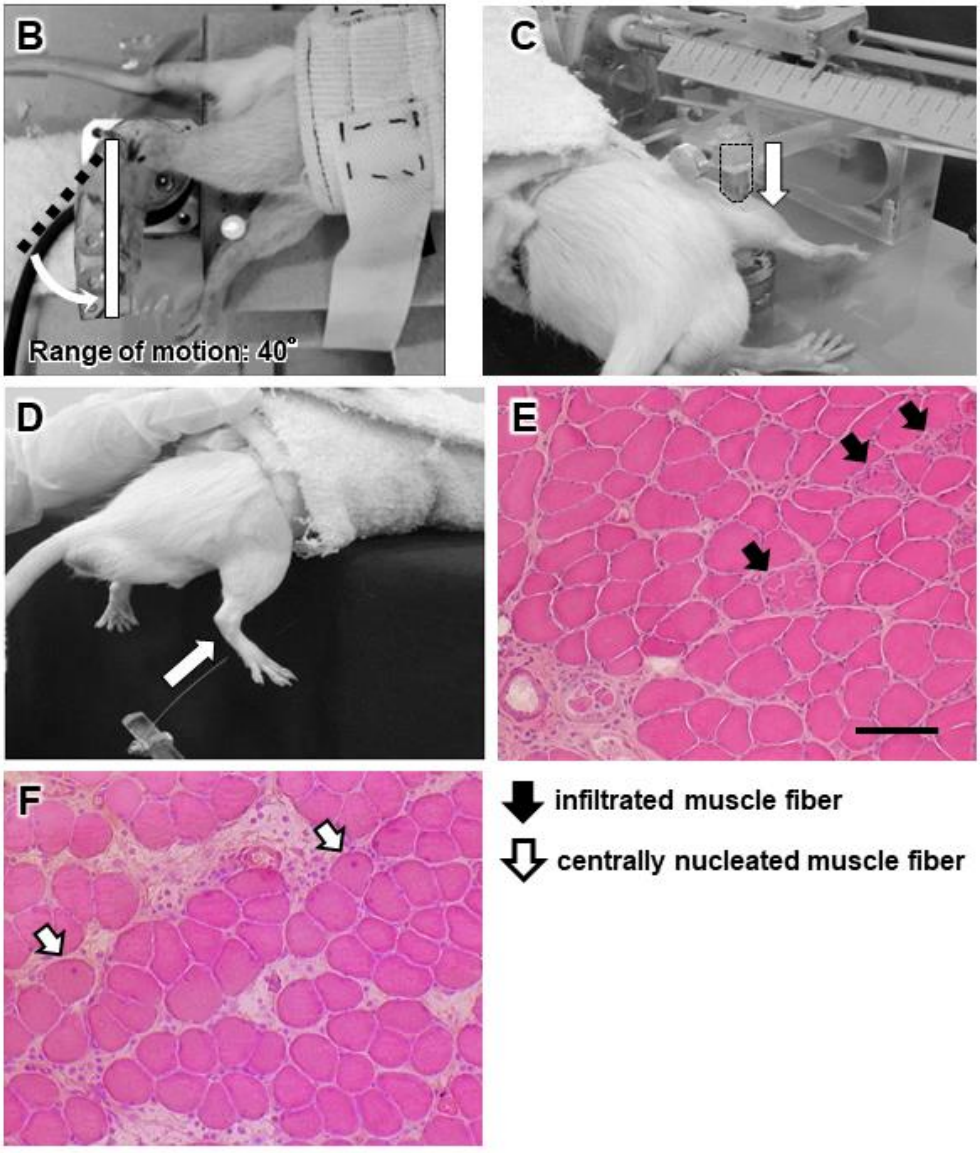
(**A**) ROM of right ankle dorsiflexion. (**B**) ROM of left ankle dorsiflexion. Horizontal axis indicates measurement time points. Data are presented as mean ± SEM (n=5 or 6). **P*<0.05 relative to associated baseline values; $^{\#}P$<0.05 relative to CON group.

Figure 5. Effects of stretching exercises on number of muscle fibers with inflammatory infiltration and central nuclei.

Histological findings were confirmed with quantitative analysis comparing age-matched naïve controls (CON, n=5), CPCP rats without cyclic stretching exercise (CPCP, n=6), and CPCP rats with cyclic stretching exercise (CPCP+STR, n=6). (**A**) Number of infiltrated muscle fibers. (**B**) Number of centrally nucleated fibers. Values are expressed as box-and-whisker plots (highest, third quartile, median, first quartile, and lowest values). Dotted lines indicate mean values. $^{\#}P$<0.05 relative to CON group.


A
Cast application
Cast removal
Reloading
Tissue sampling and H&E staining
Unloading period
Group (n)
0 weeks
4 weeks
CON (5)
Free ambulation
Free ambulation
CPCP (6)
Cast Immobilization
Free ambulation
CPCP + STR (6)
Cast Immobilization
Stretching exercise
Behavioral test
Baseline
0
1
3
5
7
10
14 days

New Figure 1



(Continued)

New Figure 1

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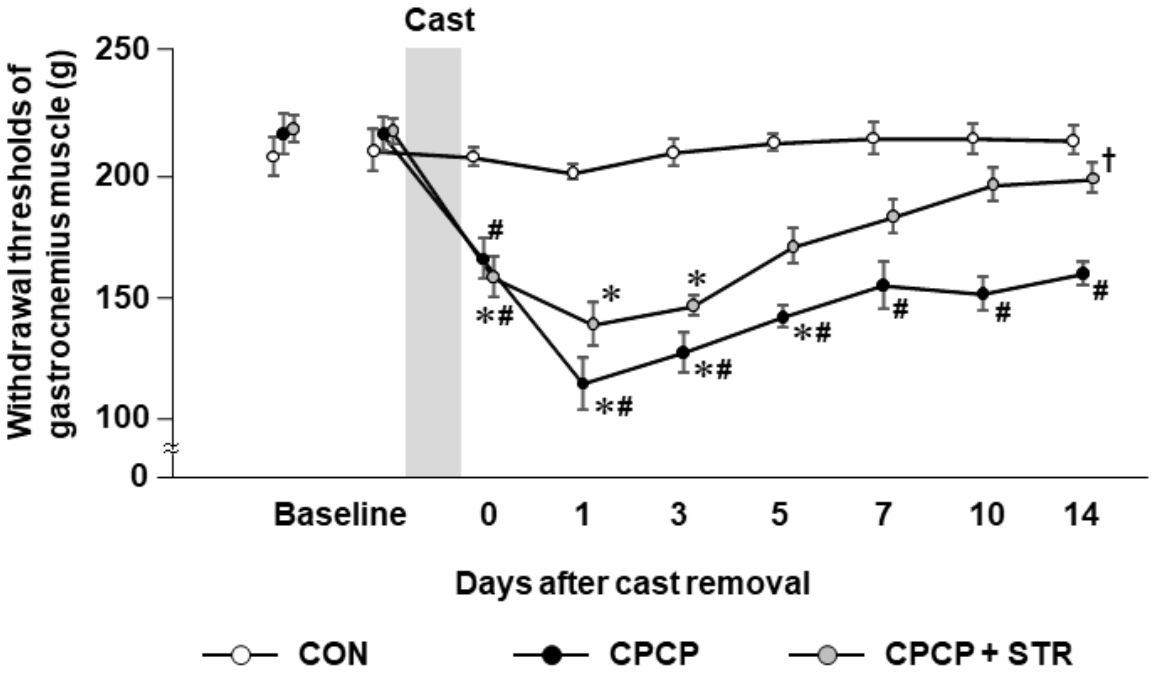
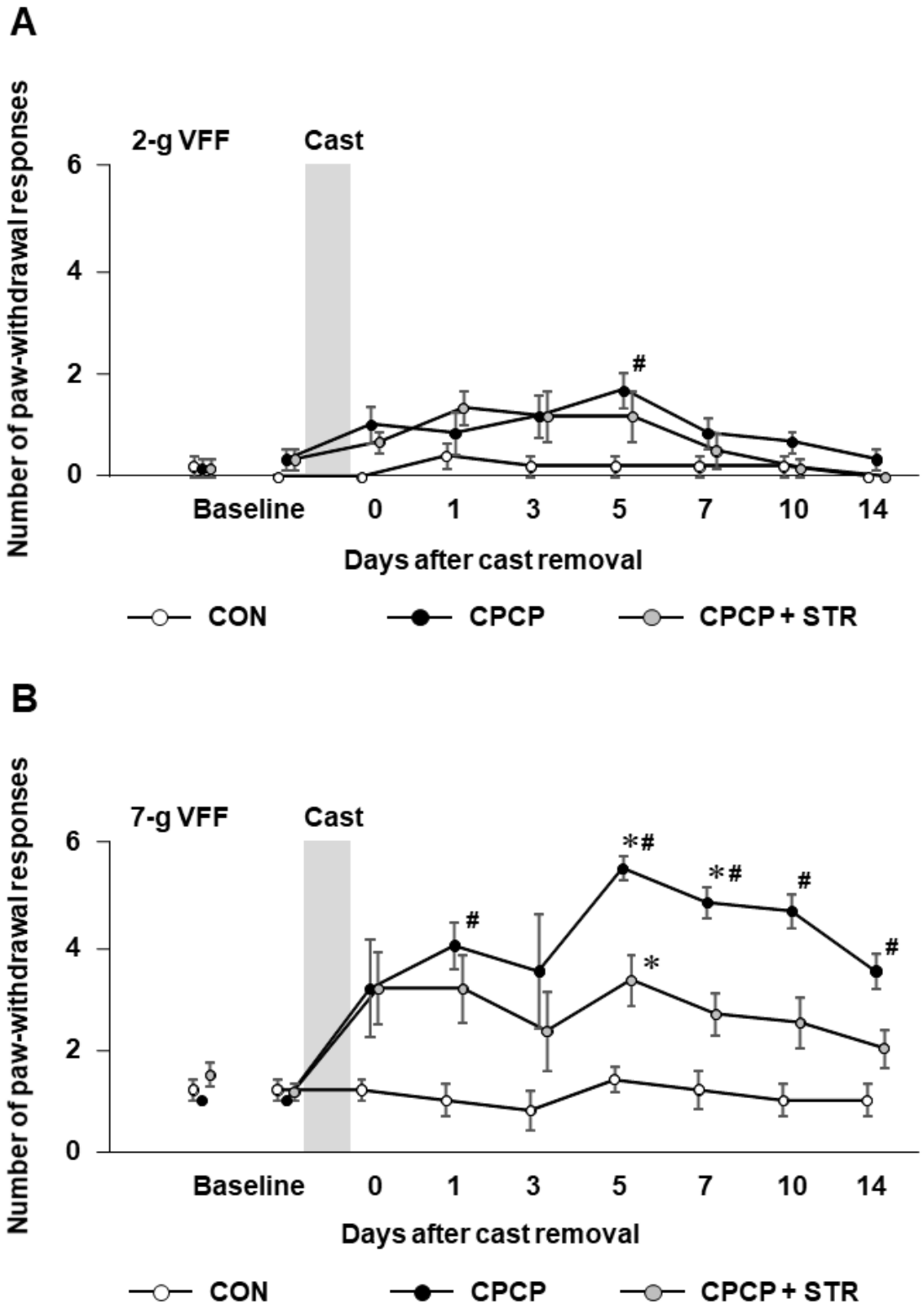

Cast
Withdrawal thresholds of gastrocnemius muscle (g)
250
200
150
100
0
Baseline
0
1
3
5
7
10
14
Days after cast removal
CON
CPCP
CPCP + STR

Figure 2



New Figure 3

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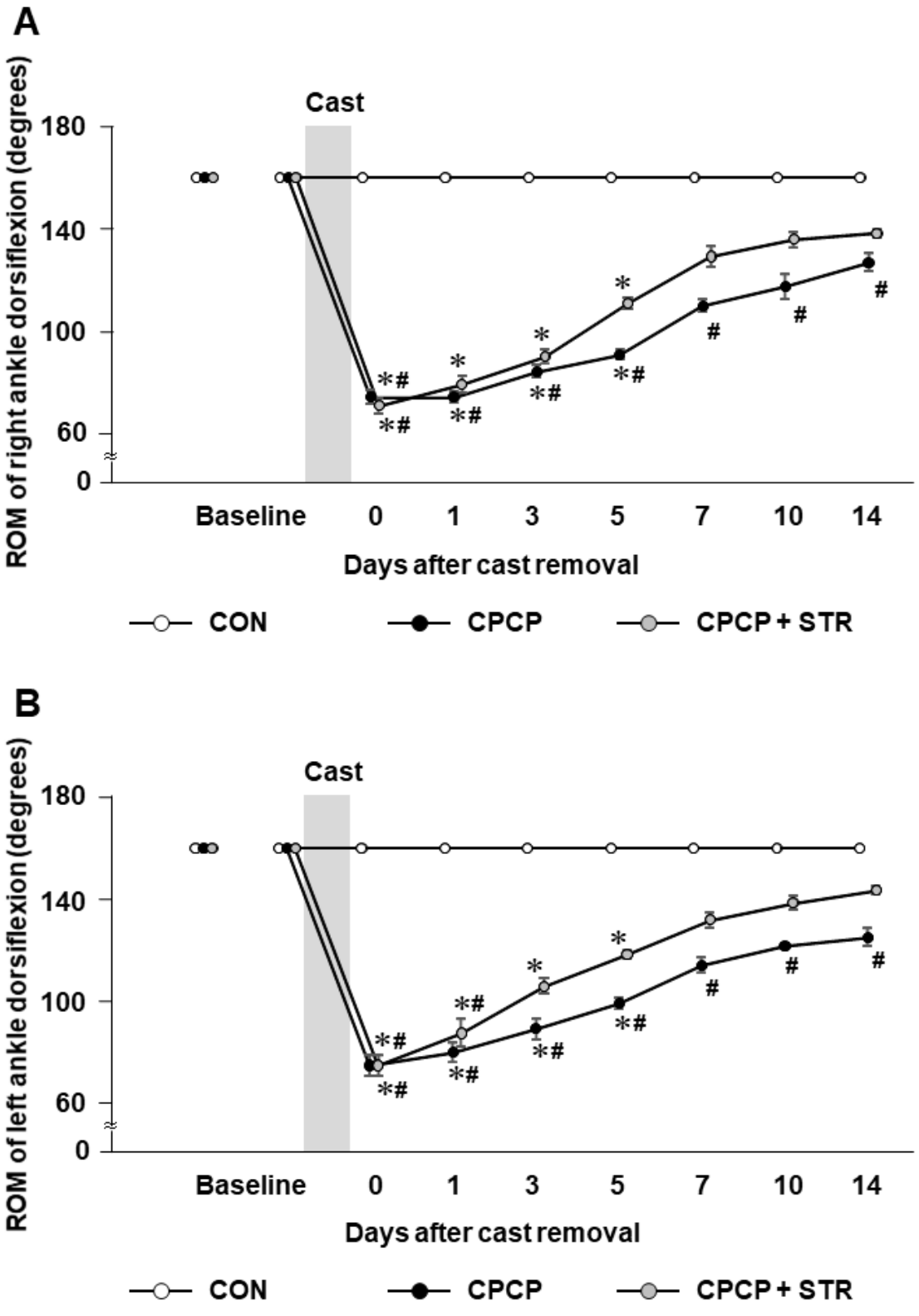

Figure 4

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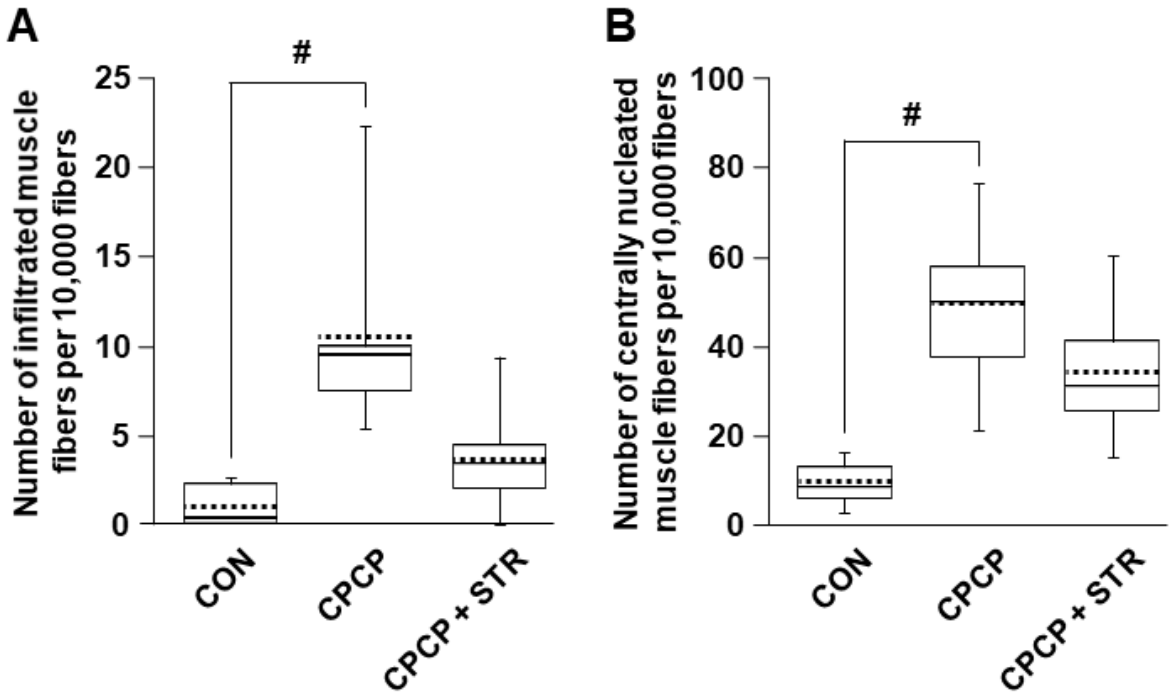

New Figure 5

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Supplementary Methods section for preliminary study

Static stretching exercise

Rats were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg Somnopentyl®; Kyoritsu Seiyaku Co., Tokyo, Japan) and the bilateral soleus muscles were stretched with the custom-built apparatus described in the current study. The amplitude of static stretches was controlled with a stepping motor (linear motor LU4B45SA-2; Oriental Motor Co. Ltd., Tokyo, Japan). Stretching exercises were performed at the day's maximum dorsiflexion angle, as measured with a goniometer. The static stretching was applied for 30 min/day, 6 days/week, beginning immediately after cast removal (prior to reloading) and continuing for 1 or 2 weeks (6 or 12 sessions total).

Myosin ATPase staining

The rats were sacrificed with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) on Day 0, 7, or 14 after cast removal and the soleus muscles from both hindlimbs of each rat were excised. Soleus muscles were embedded in optimal cutting temperature compound (TissueTek®; Sakura Finetek, Tokyo, Japan); 7-μm cross-sections were cut from the mid-portion of the muscles with a cryostat (CM1510-11; Leica, Wetzlar, Germany) and mounted on Superfrost Plus slides (Thermo Fisher Scientific, Tokyo, Japan).

Myosin ATPase staining was performed according to the protocol of Brooke

and Kaiser (1970). Briefly, sections were pre-incubated in acidic buffer (0.1 M barbital acetate and 0.1 M hydrochloride, adjusted to pH 4.6) for 5 min and then rinsed with a substrate solution (0.18 M calcium chloride and 0.1 M sodium barbital, adjusted to pH 9.4). Sections were then incubated in ATP staining buffer (0.18 M calcium chloride, 0.1 M sodium barbital, and 2.4 mM ATP disodium salt) at pH 9.4 for 45 min, washed three times in 1% calcium chloride solution for 3 min each, incubated with 2% cobalt chloride for 3 min, washed eight times with 0.01 M sodium barbital solution, and rinsed with distilled water for 2 min. Finally, sections were incubated in 1% ammonium sulfide for 1 min and rinsed with distilled water five times. Following staining, each section was sealed with Canada balsam and topped with a coverslip. Dark-stained fibers were classified as Type I (slow fibers) and light fibers as Type II (fast fibers). Type IIA fibers appeared white, whereas type IIB fibers stained gray (Lind and Kernell, 1991).

Images of the stained cross-sections were captured with an optical microscope (BZ-9000; Keyence, Osaka, Japan) at 20× magnification. The cross-sectional area of each fiber type in the soleus muscles was measured with Image J software (National Institutes of Health, Bethesda, MD, USA). More than 100 fiber measurements were recorded per animal for each type of fiber.

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Supplementary Table 1. Cross-sectional area of Type I and Type II fibers in soleus muscle of groups studied (n=5 rats [10 muscles] per group)

Fiber type	Control	IM	14 days after cast removal		
			FA	SS	CS
Type I (μm^2)	3172.4 ± 869.9 (1988.2–4755.6)	2345.4 ± 897.1 *** (1113.5–3826.5)	2817.3 ± 1052.3 ***, ### (1239.8–4555.4)	2704.7 ± 828.4 ***, ###, ††† (1574.6–4280.8)	3000.6 ± 1031.4 ***, ###, †, §§§ (1587.0–4800.4)
Type II (μm^2)	1976.3 ± 519.2 (1285.5–2963.3)	1164.8 ± 401.9 *** (635.5–1875.0)	2144.3 ± 851.6 ***, ### (926.3–3745.2)	1915.6 ± 613.8 *, ###, ††† (1155.7–3256.7)	2562.9 ± 813.0 ***, ###, †††, §§§ (1450.9–4121.8)

Values shown as mean ± SD (95% confidence interval).
IM: 4-week cast immobilization; FA: 4-week cast immobilization followed by free ambulation (free cage activity) for 14 days; SS: static stretching performed 6 times/week; CS: cyclic stretching performed 6 times/week.
Data were analyzed with the Kruskal–Wallis test followed by a Dunn–Bonferroni post-hoc test for all pairwise multiple comparisons. *p<0.05, ***p<0.001 vs. control group; ###p<0.001 vs. IM group; †p<0.05, †††p<0.001 vs. FA group; §§§p<0.001 vs. SS group.

Supplementary Table 2. Number of necrotic muscle fibers/total muscle fibers in soleus muscles in groups studied

			7 days after cast removal		
	Control (5 rats, 5 muscles)	IM (5 rats, 5 muscles)	FA (5 rats, 5 muscles)	SS (4 rats, 4 muscles)	CS (5 rats, 5 muscles)
Necrotic muscle fibers/total muscle fibers (%)	1/8131 (0.01)	31/8957 (0.34) **	201/6528 (2.99) **, ##	73/6112 (1.18) **, ##, ††	42/7456 (0.56) **, ††, §§

Values shown as number of necrotic muscle fibers/total muscle fibers (%). IM: 4-week cast immobilization; FA: 4-week cast immobilization followed by free ambulation (free cage activity) for 7 days; SS: static stretching performed 6 times/week; CS: cyclic stretching performed 6 times/week. Group comparisons were performed with a chi-square test with Bonferroni correction. $^{**}p<0.01$ vs. control group; $^{\#\#}p<0.01$ vs. IM group; $^{\dagger\dagger}p<0.01$ vs. FA group; $^{\S\S}p<0.01$ vs. SS group.