Effects of hypertonia on contracture development in rat spinal cord injury

Running Title: Contracture and hypertonia

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Abstract

Study design: Experimental animal study.

Objectives: Spastic hypertonia is originally believed to cause contractures from clinical observations. Botulinum toxin is effective for the treatment of spasticity and is widely used in patients who have joints with contractures. Using an established rat model with knee contractures after spinal cord injuries, we aimed to verify whether hypertonia contributes to contracture development, and the botulinum toxin improves structural changes in muscles and joint components responsible for contractures.

Setting: University laboratory in Japan.

Methods: To evaluate the effect of hypertonia on contracture development, the rats received botulinum toxin injections after spinal cord injuries. Knee extension motion was measured with a goniometer applying a standardized torque under anesthesia, and the contribution by muscle or non-muscle structures to contractures were calculated by measuring joint motion before and after the myotomies. We quantitatively measured the muscle atrophy, muscle fibrosis, and synovial intima length.

Results: Botulinum toxin injections significantly improved contractures, whereas did not completely prevent contracture development. Botulinum toxin was effective in improving the muscular factor, but little difference in the articular factor. Spinal cord injuries induced muscle atrophy, and botulinum toxin significantly accelerated muscle atrophy and fibrosis. The synovial intima length decreased significantly after spinal cord injuries, and botulinum toxin did not improve this shortening.

Conclusions: This animal study provides new evidence that hypertonia is not the sole cause rather is the partial contributor of contractures after spinal cord injuries. Furthermore, botulinum toxin has adverse effects in the muscle.

INTRODUCTION

Joint contractures are major complications of spinal cord injuries (SCI)[1, 2], and are characterized by limitations in the passive range of motion (ROM) of the affected joints[3]. Decreased ROM limits activities of daily living for SCI patients, and predisposes them to other complications such as pressure sores[1]. Positioning, stretching, and physical therapy are advocated to prevent and treat contractures. The usefulness of passive joint motion exercises has been validated in many clinical studies that determined their therapeutic efficacy[2]. Nevertheless, we are often confronted with SCI patients who have contractures that limit limb function.

Based on clinical observations, spastic hypertonia is believed to cause contractures after central nervous system injuries for many years[3]. We have previously established SCI rat models with knee contractures[4] and proposed that both muscular and articular factors contribute almost equally to the overall progression of the contractures after 14 days of SCI[5]. Subsequently, we have shown that the intra-articular alterations after SCI exhibit the specific changes that differed from those observed in animal models with immobilized joints[6-8]. However, what causes the SCI-specific characteristics of joint contractures has not been previously investigated, and whether hypertonia plays a part in contracture development after SCI remains controversial.

Botulinum toxin (BTX) induces a reversible muscle relaxation by inhibiting acetylcholine release from the presynaptic terminals of the neuromuscular junction in the peripheral nervous system[9]. Clinically, BTX is considered safe and effective for the treatment of spasticity[9], and it has been widely used in patients with SCI[10], celebral palsy[11], and cerebrovascular disease[12]. The BTX injection also improves the limitations in ROM and the functional outcome[10], but it appears to be merely a consequence of muscle relaxation, not due to ameliorations of structural alterations. The effect of BTX injections on

histopathological changes in muscles and periarticular structures have not been understood.

In this study, using an established SCI model with contractures, we aimed to verify whether (1) hypertonia contributes to contracture development and (2) the BTX injection improves structural changes in muscles and joint components responsible for contractures after SCI.

METHODS

Experimental Design

Total 20 male Wistar rats (SLC Japan Inc., Shizuoka, Japan), 10 weeks old, were used in this study. These rats were randomly divided into the following 3 groups: a healthy group that had no intervention (control group, n = 6 rats), an untreated group with SCI (SCI group, n = 6 rats), and a BTX injection group after SCI (BTX group, n = 8 rats). Knee flexion contractures develop in rats with SCI for first 14 days postinjury[4]. Therefore, to evaluate the effect of hypertonia on contracture development, the rats in the BTX group received injections at either the immediate or 14th postoperative day (n = each 4 rats). The rats at 14 and 28 days after the injections were evaluated and compared with the age-matched animals in the control and SCI groups (Figure 1). The right and the left knee joint served as different samples. The sample sizes were calculated by a power analysis based on pilot results in order to detect a 10º difference in ROM 19 times out of 20[5].

Surgical procedures and postoperative care conformed to those in our previous studies[4-8]. Rats in the SCI and BTX groups were anesthetized by an intraperitoneal administration of 40 mg/kg of sodium pentobarbital and subcutaneously injected with 0.02 mg/kg buprenorphine to give relief of pain. Then, their spinal cords were transected completely at the T8 level. This procedure led to the development of knee joint flexion contractures[4, 5]. Postoperative pain was controlled with 0.05mg/kg of buprenorphine given

subcutaneously every 8 to 12 hours for the first 72 hours. The rats were housed in sterilized cages with bedding (cedar shavings), and were maintained under artificial conditions at 22 ± 1 °C and a cycle of 12h of light and 12h of dark. The animals had free and easy access to food and tap water, and unlimited activity. The behavior of all animals was observed every day throughout the experimental period. Before surgery and at the end of each timepoint, withdrawal reactions to stimuli (extension, pain, and pressure) were evaluated.

BTX Injections

The lyophilized clostridium botulinum toxin type A neurotoxin complex (BOTOX; Allergan, Coolock, Dublin, Ireland) was diluted with saline to a final concentration of 1 UI/ml. BTX solution was injected into both sides of all knee flexors (biceps femoris, semitendinosus, semimembranosus, gastrocnemius, and gracilis) of the rats in the BTX group (0.2 UI/ml was given in each muscle). This dose of BTX per muscle has been shown previously to produce muscle relaxant for 14 days[13, 14]. Therefore, the rats evaluated at 42 days after SCI were injected once every 14 days (Figure 1).

ROM Measurements

ROM measurements of knee extension were performed under anesthesia with isoflurane according to the previously described method[5, 15, 16]. Briefly, knee motion in extension was measured with a mechanical goniometer applying a standardized torque (0.06 Nm) by intervals of 1°. The lateral femoral condyle was the pivot point while the femur was fixed, and extension moments were applied to the tibia. The degree of the limitation in ROM was assessed by measuring the femorotibial angle. Normal extension ROM of healthy rats is approximately 15°[4, 5]. The measurements were done blindly by 2 investigators and repeated them 5 times for each knee. Values were the mean of the 10 measurements, the combined

measurements by both investigators.

The animals were killed by exsanguination. Myotomies of the trans-articular muscles were then performed, and ROM was measured again in extension. Measurements after myotomies were completed within 15min of the animal's deaths, in order to minimize the possibility of postmortem rigidity. The muscular factor that contributes to contractures was defined as limited ROM in the muscles including tendon and fascia, and the articular factor was defined as limited ROM in the articular components (bone, cartilage, synovium, capsules, and ligaments)[17]. According to our previous method[15, 16], the formulas assessed by measuring ROM before and after the myotomies allow isolation of the muscular and articular factors responsible for contractures and are as follows: muscular factors = ROM no myotomy − ROM after myotomy (within each group); articular factors = ROM after myotomy of each group − ROM after myotomy of the control group.

Sampling and Histological Preparation

The biceps femoris muscles, which are the biggest in rat knee flexors and therefore contribute to the development of knee flexion contractures, were harvested and the ratio of skeletal muscle wet weight to whole-body weight was calculated. Frozen sections 10 μm thick were cut from muscle samples and were then stained with hematoxylin and eosin. Muscle fiber cross-sectional area were measured in over 100 muscle fibers from each muscle in each animal. After the knee joints and surrounding soft tissue were harvested, standardized 5 μm sections were obtained at the medial midcondylar level in the sagittal plane following a previously published method of Kawamoto[18]. The sections of each knee were stained with hematoxylin and eosin.

Quantification of Fibrosis in Muscle Tissue

We assessed muscular fibrosis leading to poor muscle extensibility in muscular contracture by slight modification of the method according to Hadi *et al*.[19]. The histologic sections from the biceps femoris muscles were stained with picrosirius red. Fibrosis was quantified for an average of 10 images obtained at 10× magnification randomly chosen at the middle third of each section. The yellow color of the muscle cells and the red color of the connective tissue were identified on the sections. The area of each color was measured separately with Image Tool software (Image J 1.47v; National Institutes of Health, Bethesda, MD, USA), and the percentage of connective tissue area was then calculated.

Quantification of Synovial Intima Length

To evaluate the shortening of the joint capsule responsible for articular contracture, we measured posterior synovial intima length as described by Ando *et al*.[20]. The synovial lining contour from the synovio-cartilage junction of the femur or tibia to the posterior horn of the meniscus was traced with Adobe Photoshop CS2 (Adobe Systems Inc, San Jose, CA, USA), and then its length was measured with Image Tool software. The length of the superior and inferior subdivisions of the synovial intima in the posterior joint capsule were summed to provide the total length of the synovial intima.

Statistical Analyses

The results for ROM, muscle wet weight, muscle fiber cross-sectional area, muscle fibrosis, and synovial intima length were analyzed statistically with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)[21]. The results were compared among all groups within timepoint using analysis of variance following Tukey's honestly significant difference test. An alpha of less than 0.05 was chosen as the significance level for all

171 statistical analyses.

172 Statistical analyses of muscular and articular factors in contractures were conducted
173 with Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). ROM data from
174 each condition were averaged for each group in the muscular and articular factors. The
175 standard deviations (SD) on muscular and articular factors estimates were derived according
176 to a method of calculating SD for differences in group means[22]. We calculated the SD for the
177 mean differences among the groups and 95% confidence intervals (CI) were estimated.
178 When the length of the 95% CI of the between-group mean difference did not overlap zero,
179 we identified muscular and articular factors contributed significantly to contracture
180 development[5, 15-17].

181

182 **RESULTS**

183 **Functional Outcome**

184 The rats with SCI demonstrated complete flaccid paraplegia after injury, and thereafter
185 showed kick movements, clonic and high-frequency flexion-extension movements, and
186 hyperreflexia which were characteristic in spasticity[23]. Meanwhile, the rats after the BTX
187 injection showed the reflex response to stimuli, but kick movements and hyperreflexia were
188 not observed. This result indicated that the dose of BTX was adequate to suppress spasticity
189 after SCI.

190

191 **Limitations in ROM**

192 The limitation in knee extension ROM developed significantly in rats with SCI (Figure 2 and
193 Supplementary Table 1). BTX injections significantly improved the limitation in ROM after
194 SCI (Figure 2 and Supplementary Table 1), whereas BTX did not completely prevent the
195 development of limitations in ROM after SCI (Figure 2 and Supplementary Table 1).

196

197 **Muscular and Articular Factors of Contractures**

198 BTX injections significantly improved the muscular factor at each timepoint, when
199 compared to the SCI groups (Table 1). In the articular factor, no differences were found
200 between the SCI and BTX groups, except for the timepoint of the BTX injection at day 0 and
201 evaluation after day 28 of SCI (Table 1).

202

203 **Changes in the Muscle**

204 Microscopic findings showed that SCI induced muscle atrophy (Figure 3A-J). Likewise, the
205 muscle fiber cross-sectional area was decreased significantly after SCI compared to the
206 control group, although no statistically significant differences were found between the SCI
207 and BTX groups (data not shown). In addition, the muscle wet weight decreased
208 significantly after SCI, and BTX group was more prominent (Figure 3K and Supplementary
209 Table 2). BTX injections significantly accelerated muscle fibrosis and increased muscle
210 fibrosis at day 28 and 42 (Figure 3L and Supplementary Table 3).

211

212 **Changes in the Joint Capsule**

213 Microscopic findings showed that adhesions between synovial fold and the synovial
214 membrane of the posterior joint capsule were observed after SCI, although there were no
215 differences between the SCI and BTX groups (Figure 4A-J). In line with these observations,
216 the synovial intima length decreased significantly after SCI, and BTX injections did not
217 improve this shortening (Figure 4K and Supplementary Table 4).

218

219 **DISCUSSION**

220 Our study had 2 objectives: to verify whether hypertonia contributes to contracture

development after SCI and to examine the effects of BTX on contracture-associated alterations of muscles and joint components. The widely held belief that spastic hypertonia causes joint contracture was not always true. The results presented here suggest that hypertonia is the partial contributor rather than the sole cause of contractures after SCI. BTX injections significantly improved contractures, but did not completely prevent contracture development. Moreover, the findings indicated that BTX injections significantly improved the muscular factor responsible for contractures, whereas BTX induced marked muscle atrophy and accelerated fibrosis. In contrast, BTX had no effect on articular contractures.

Spastic hypertonia is originally believed to cause contractures following paralysis from clinical observations[2, 3]. In an attempt to elucidate experimentally this clinical observation, BTX was injected into all the knee flexors immediately after SCI and then suppressed hypertonia. Consequently, BTX did not prevent contracture development after SCI. On the other hand, we have reported previously that knee flexion contractures developed in rats with SCI for first 14 days postinjury[5], and therefore we investigated the effect of treatment with BTX by injections at the 14th postoperative day. Thus, BTX had treatment effects on joint ROM limitations. We also found little differences between the results of ROM at the immediate and 14th postoperative day. This would indicate that the improvement in ROM limitations by BTX injections may be due to treatment effects, but not preventive effects. Overall, our findings cast doubt whether only hypertonia causes joint contracture.

The muscle fibrosis is closely related to contracture development after joint immobilization[24], and therefore we evaluated the fibrosis. Although there was not a significant increase in muscle fibrosis after SCI, BTX significantly induced the fibrosis with longer duration after the injections. BTX induces the time- and dose-dependent increases in type I and III collagens, IGF-1, and TGF-β expression associated with fibrosis[25] and

246 vimentin staining, a marker for fibrosis, in rabbit muscles[26]. Similarly, in human extraocular
247 muscles, BTX induces muscle fibrosis[27]. These earlier reports support the present results.
248 Besides, our results demonstrated that muscle atrophy occurred in animals with SCI and was
249 more marked by BTX injections. Muscle atrophy is a well-known phenomenon observed in
250 SCI patients[28], and BTX injections commonly induce human muscle atrophy[29]. Our findings
251 in the rat model with SCI closely reflect these outcomes in humans. Additionally, the passive
252 stiffness decreases in atrophied muscle by BTX injections[30]. Taken together, improved
253 muscular contractures by BTX injections in this study may not result from improvements in
254 structural changes of muscles.

255 The shortening of the synovial intima length proved an excellent marker for the
256 incidence and severity of knee flexion contractures after SCI[8, 16] or immobilization[20]. This
257 led us to conclude that BTX injections into muscles had no effect on articular contractures.
258 BTX is an agent inhibiting acetylcholine release from the neuromuscular junction in the
259 peripheral nervous system[9]; thus this explanation may be quite plausible. Previously, we
260 reported that joint movement (ie, mechanical stimuli) are crucial for improvements in
261 articular alterations causing contractures after SCI[15, 16]. The present findings are also in line
262 with our previous results. Both muscular and articular factors contribute almost equally to
263 the overall progression of the contractures after 14 days of SCI[5]. Indeed, BTX improved
264 muscular factors, even apparent improvements; however, ROM in the BTX group did not
265 recover to the same range as that in the control group, indicating that articular factors are
266 critically involved in the contracture development.

267 Our study has several limitations. First, we used the right and the left knee joint
268 served as different samples from same rats. The use of both joints has the advantages of
269 minimizing the number of experimental animals needed for ethical reasons and providing
270 equivalency of sample size for statistical purposes. However, its use cannot preclude chance

findings attributable to intra-animal and inter-animal variation. That is, outcomes in one knee is likely to be very similar to outcomes from the other knee because both knees belong to the same rat. The lack of accounting for the correlation between knees belonging to the same rat produces confidence intervals that are narrower than what they should be. The second limitation is that we assessed only histologic changes responsible for contractures and did not analyze other factors (eg, mRNA and protein levels) involved in structural changes in muscles and joint components. Finally, and most importantly, we did not examine spasticity using electromyography more specifically. Thus, the question remains as to whether spasticity directly contributes to contracture development. There are possible other factors may cause contracture or mediate the effect of spasticity on contracture. In addition, to clarify the direct effect of BTX, it may be necessary to administer BTX to healthy rats without contractures, although the effect of BTX on normal muscle is well documented.

In conclusion, here we provide new evidence that hypertonia is not the sole cause rather is the partial contributor of contractures. Furthermore, in the treatment aimed to improve spasticity and/or contracture after SCI, BTX can suppress spasticity but has adverse effects of muscle atrophy and fibrosis; thus may be less predictably true effective against contractures.

Data archiving

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Statement of Ethics

This study was approved by the Institutional Animal Care and Use Committee (Permission number: P130408) and carried out according to the Kobe University Animal Experimentation Regulations.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

HM was responsible for designing and directing the protocol, interpreting results, and writing the manuscript. JO was responsible for designing and directing the protocol, interpreting results, and revising the manuscript. TY, SI, and TW was responsible for conducting the experiment, extracting and analyzing data, and revising the manuscript. NK, YS, and TA was responsible for designing the protocol, interpreting results, and revising the manuscript.

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Legend for Supplementary Material

Supplementary Table 1 Knee extension ROM in each group. The results of ROM measurements at each timepoint are presented as mean ± 1SD. F statistic, degrees of freedom, 95% CI, p values, and power when compared among all groups within timepoint are shown.

Supplementary Table 2 The wet weight of the biceps femoris muscle in each group. The results of muscle wet weight at each timepoint are presented as mean ± 1SD. F statistic, degrees of freedom, 95% CI, p values, and power when compared among all groups within timepoint are shown.

Supplementary Table 3 The fibrosis of the biceps femoris muscle in each group. The results of muscle fibrosis at each timepoint are presented as mean ± 1SD. F statistic, degrees of freedom, 95% CI, p values, and power when compared among all groups within timepoint are shown.

Supplementary Table 4 Posterior synovial intima length in each group. The results of posterior synovial intima length at each timepoint are presented as mean ± 1SD. F statistic, degrees of freedom, 95% CI, p values, and power when compared among all groups within timepoint are shown.

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421 **Figure Legends**

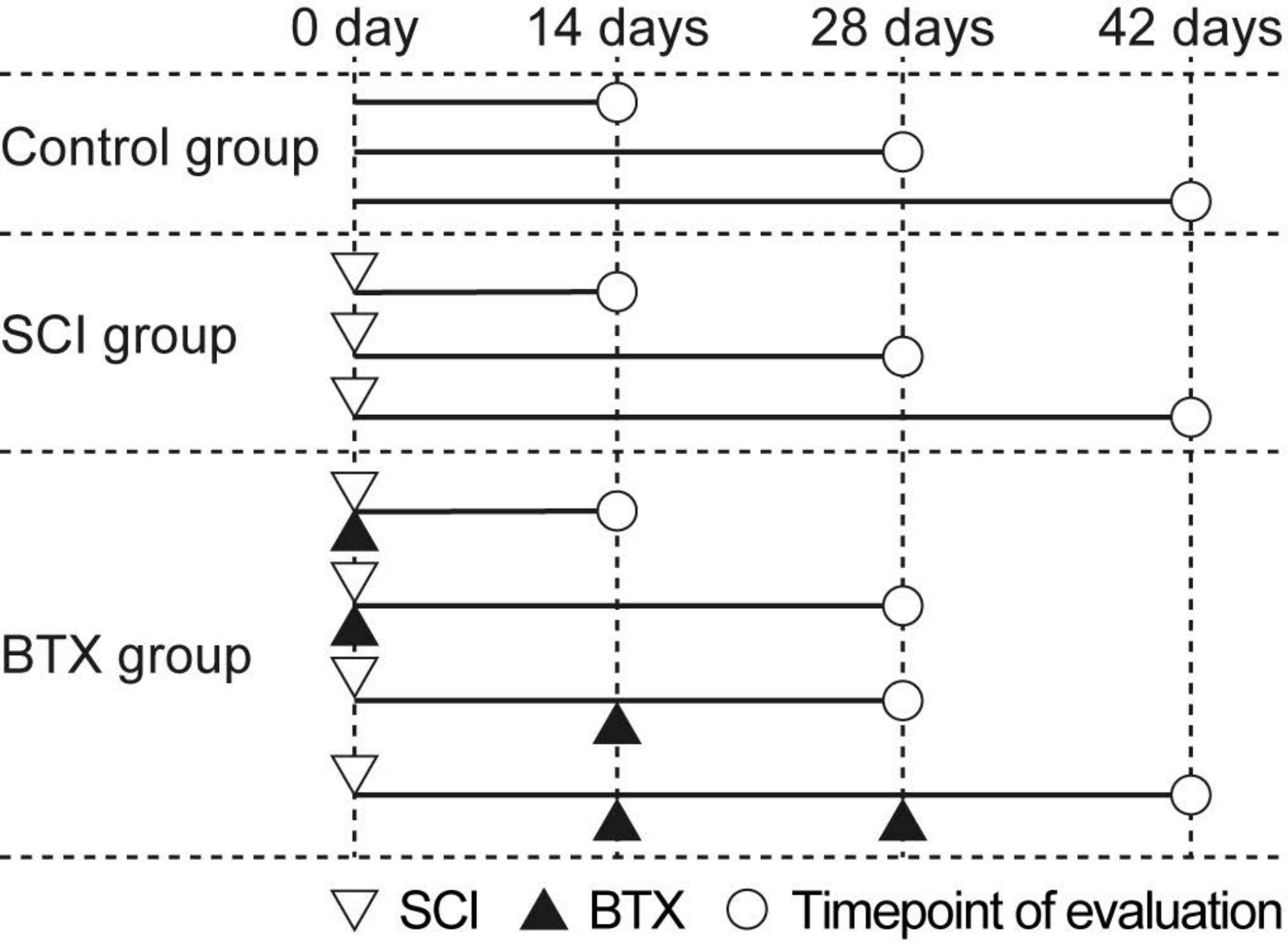
422 **Figure 1** A diagram of the experimental design is shown. The rats were randomly divided
423 into the control, SCI, and BTX groups (n = 2 rats per timepoint). The rats of the BTX group
424 received BTX injections into both sides of all knee flexors at 0, 14 or at 28 days after SCI,
425 and were evaluated and compared with the age-matched animals in the control and SCI
426 groups at 14, 28 or 42 days respectively after SCI. The right and the left knee joint from
427 same animal served as independent samples (n = 4 knees from 2 rats per timepoint for all
428 groups).

429 **Figure 2** The graph shows knee motion measured for extension with a goniometer. Two
430 approaches to BTX injections were evaluated: BTX injections was provided at the
431 immediate after SCI (BTX 0) and BTX injections was given on the 14th day postoperatively
432 (BTX 14). Data are presented as the mean ± SD. * Indicates a significant difference when
433 compared to the age matched control. † Indicates a significant difference between the SCI
434 and BTX groups. Four knees from each group were evaluated at each timepoint.

435 **Figure 3** Representative photomicrographs show the biceps femoris muscles stained with
436 picrosirius red in the control (A-C), SCI (D-F), and BTX (G-H) groups at each timepoint.
437 Two approaches to BTX injections were evaluated: BTX injections was provided at the
438 immediate after SCI (BTX 0) and BTX injections was given on the 14th day postoperatively
439 (BTX 14). Scale bars = 100 μm. The graphs show the muscle wet weight (G) and the muscle
440 fibrosis (H) in each group. Data are presented as the mean ± SD. * Indicates a significant
441 difference when compared to the age matched control. † Indicates a significant difference
442 between the SCI and BTX groups. Four knees from each group were evaluated at each
443 timepoint.

444 **Figure 4** Representative photomicrographs show the synovial membrane in the posterior
445 capsule around the femur stained with hematoxylin and eosin in the control (A-C), SCI (D-

446 F), and BTX (G-H) groups at each timepoint. Two approaches to BTX injections were
447 evaluated: BTX injections was provided at the immediate after SCI (BTX 0) and BTX
448 injections was given on the 14th day postoperatively (BTX 14). Scale bars = 1 mm. The
449 graph shows the posterior synovial intima's length in each group (K). Data are presented as
450 the mean ± SD. * Indicates a significant difference when compared to the age matched
451 control. Four knees from each group were evaluated at each timepoint.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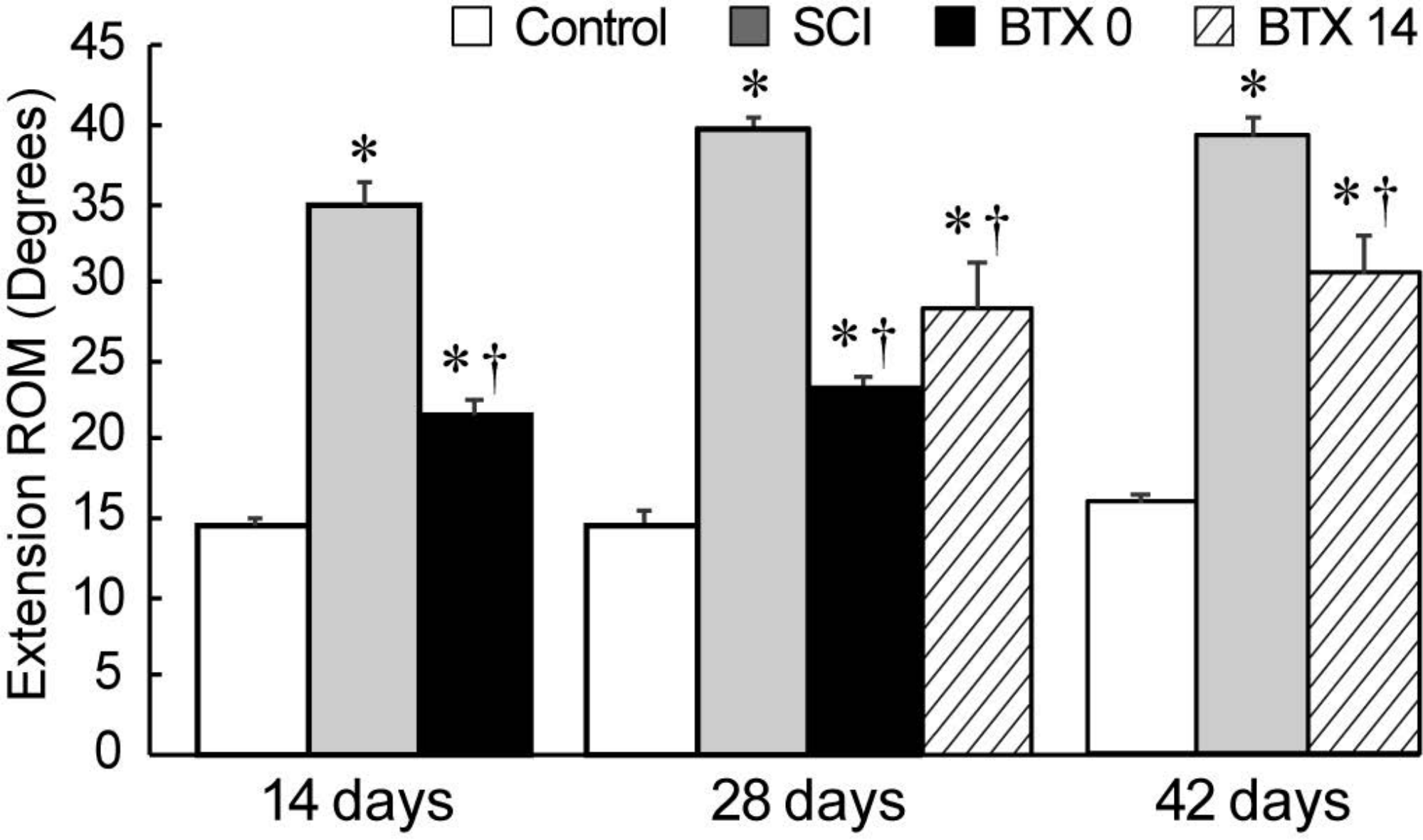
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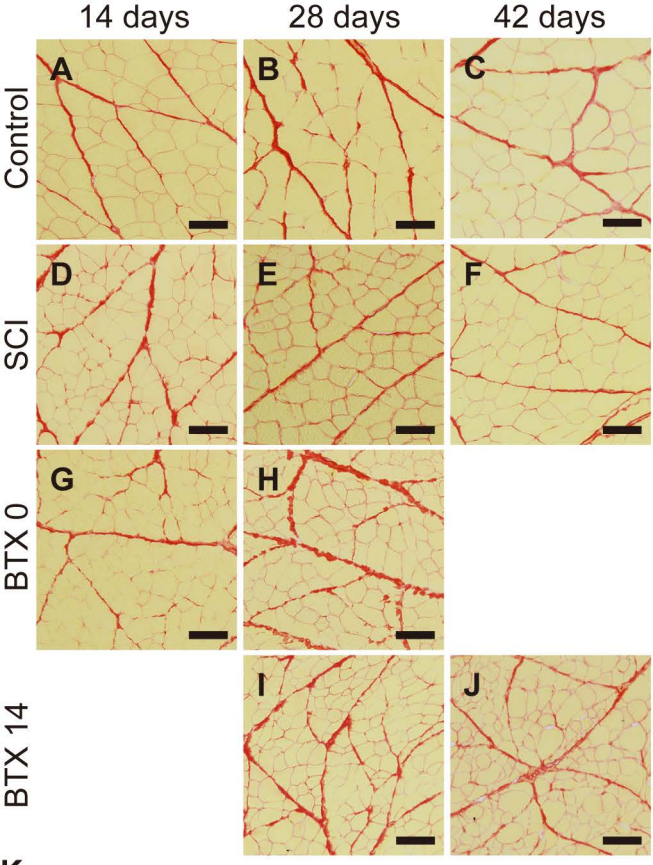
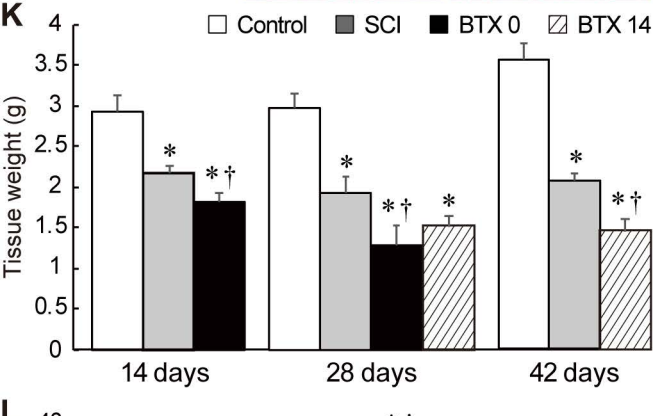
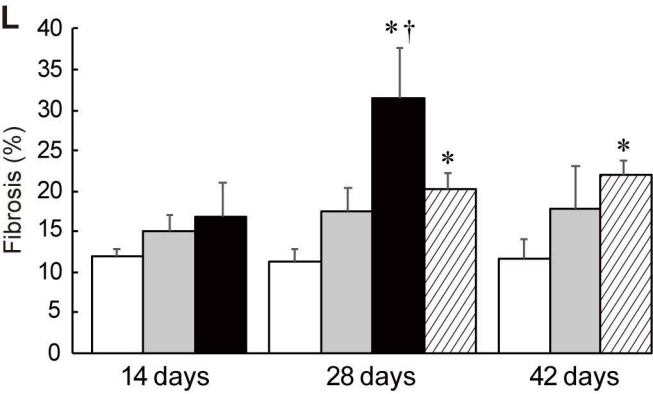
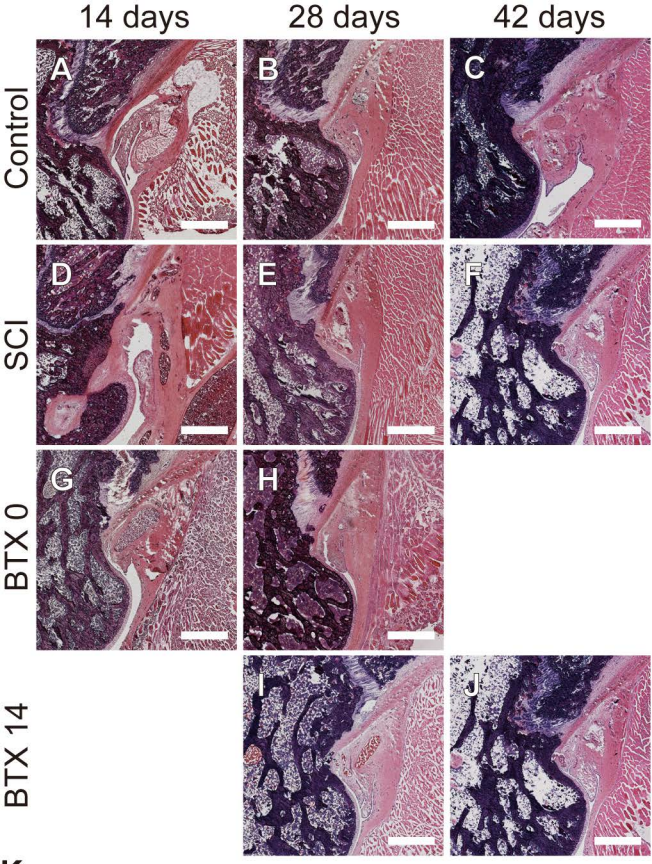
28 days
42 days


14 days
28 days
42 days
Control
SCI
BTX 0
BTX 14
A
B
C
D
E
F
G
H
I
J


K
Control
SCI
BTX 0
BTX 14
Tissue weight (g)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*†
14 days
28 days
42 days


L
Fibrosis (%)
40
35
30
25
20
15
10
5
0
*†
*
14 days
28 days
42 days


14 days
28 days
42 days
Control
SCI
BTX 0
BTX 14
A
B
C
D
E
F
G
H
I
J

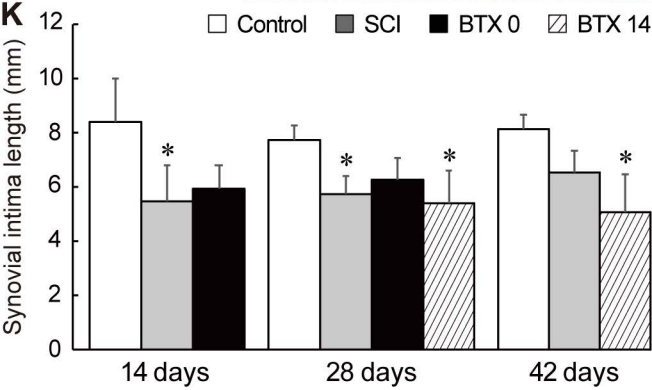

K
Control
SCI
BTX 0
BTX 14
Synovial intima length (mm)
0
2
4
6
8
10
12
*
14 days
28 days
42 days

Table 1 Muscular and articular factors of contractures

Timepoint	Muscular factor*													Articular factor*										
	ROM (mean ± 1SD°)				Percentage (%)†				95% CI					ROM (mean ± 1SD°)				Percentage (%)†				95% CI				
	Control	SCI	BTX 0	BTX 14	Control	SCI	BTX 0	BTX 14	Control vs SCI	Control vs BTX 0	SCI vs BTX 0	Control vs BTX 14	SCI vs BTX 14	Control	SCI	BTX 0	BTX 14	Control	SCI	BTX 0	BTX 14	Control vs SCI	Control vs BTX 0	SCI vs BTX 0	Control vs BTX 14	SCI vs BTX 14
14 days	5.0 ± 1.0	14.8 ± 1.0	1.7 ± 0.6	—	—	58.6 ± 2.2	13.5 ± 4.4	—	-11.55 to -8.15‡	1.91 to 4.69‡	11.75 to 14.55‡	—	—	—	10.5 ± 0.9	10.5 ± 0.5	—	—	41.4 ± 2.2	86.5 ± 4.4	—	—	—	-1.33 to 1.28	—	—
28 days	5.4 ± 0.6	19.4 ± 1.3	5.9 ± 1.8	9.8 ± 2.4	—	63.4 ± 4.1	41.0 ± 6.4	49.8 ± 7.2	-15.80 to -12.30‡	-2.82 to 1.77	10.86 to 16.19‡	-7.43 to -1.37‡	6.33 to 12.97‡	—	11.2 ± 1.3	8.2 ± 0.8	9.6 ± 1.0	—	36.6 ± 4.1	59.0 ± 6.4	50.2 ± 7.2	—	—	1.13 to 4.87‡	—	-0.45 to 3.65
42 days	5.9 ± 1.7	16.4 ± 2.6	—	8.8 ± 2.7	—	55.8 ± 6.8	—	42.3 ± 8.7	-14.22 to -6.68‡	—	—	-6.69 to 1.04	3.10 to 12.15‡	—	12.9 ± 1.7	—	11.7 ± 1.8	—	44.2 ± 6.8	—	57.7 ± 8.7	—	—	—	—	-1.90 to 4.25

*Muscular and articular factors were calculated according to the formulas in the Methods section.

†Muscular and articular proportions were expressed as percentages, when the total factors were 100% at each time.

‡Significant difference, using a length of the 95% CI not overlapping zero.

BTX 0 were received BTX injections at the immediate after SCI.

BTX 14 were received BTX injections at the 14th day after SCI.