

1 Effects of a four-week static stretch training program on passive stiffness of human
2 gastrocnemius muscle-tendon unit in vivo.

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8
9 **Abstract**

10 Static stretch is commonly used to prevent contracture and to improve joint mobility.
11 However, it is unclear whether the components of the muscle-tendon unit are affected
12 by a static stretch training program. This study investigated the effect of a four-week
13 static stretch training program on the viscoelastic properties of the muscle-tendon unit
14 and muscle. The subjects comprised eighteen male participants (mean age: 21.4 years \pm
15 1.7). The range of motion (ROM), passive torque, muscle-tendon junction (MTJ)
16 displacement and muscle fascicle length of the gastrocnemius muscle were assessed
17 using both ultrasonography and a dynamometer whilst the ankle was passively
18 dorsiflexed. After the initial test, the participants were assigned either to a group that
19 stretched for four-week (N = 9) or to a control group (N = 9). The tests were repeated
20 after the static stretch training program.

21 ROM and MTJ displacement significantly increased, and the passive torque at 30 deg
22 significantly decreased, in the stretching group after the study period. However, there
23 was no significant increase in muscle fascicle length. These results suggest that a
24 four-week static stretch training program changes the flexibility of the overall MTU
25 without causing concomitant changes in muscle fascicle length.

26
27 **Key words:**

28 Static stretch, Long-term effects, Ultrasonography, Muscle tendon unit, Gastrocnemius

1 Introduction

2
3 Static stretch (SS) is commonly employed to prevent contracture and to improve joint
4 mobility. There have been many studies about the effects of SS training programs (Chan
5 et al. 2001; Covert et al. 2010; Gajdosik 1991, 2001; Marques et al. 2009; Reid and
6 McNair 2004; Santonja Medina et al. 2007), and in these studies SS training programs
7 were shown to have increased the maximum range of motion (ROM). However, the
8 measurement of maximum ROM has a number of limitations. For example, the method
9 is influenced by factors such as pain, stretch tolerance and reflex activation of the
10 agonist muscle (Magnusson et al. 1996a; McHugh et al. 1998). It has been pointed out
11 that an alternative approach to assess the passive torque would be useful for determining
12 the stiffness of overall muscle-tendon unit (MTU) (Toft et al. 1989).

13 Many studies have indicated that SS training decreased MTU stiffness, and
14 changed MTU viscosity and elasticity (Feland et al. 2001; Kubo et al. 2002; Mahieu et
15 al. 2007; Willy et al. 2001). On the other hand, it has also been reported that changes
16 occurred in maximum ROM, but not in MTU stiffness, after 3- and 8-week SS training
17 programs (Ben and Harvey 2010; Bjorklund et al. 2001; Folpp et al. 2006; Magnusson
18 et al. 1996; Ylinen et al. 2009). Weppeler and Magnusson (2010) pointed out that the
19 improvement in maximum ROM at the conclusion of the SS training program was
20 predominantly due to a modification in stretch tolerance (i.e. subjects' sensation). Thus,
21 it is still unclear whether any decrease in MTU stiffness after stretching can be
22 attributed to alterations in the muscle, or alterations in another component of the
23 viscoelastic MTU.

24 Morse et al. (2008) reported that muscle and tendon stiffness could be estimated
25 using ultrasonography to measure the movement of the myotendinous junction (MTJ)
26 during passive stretching. In addition, they reported that there was a decrease in the
27 stiffness of the overall MTU and muscle (no change was apparent in the tendon)
28 immediately after a five-minute SS session. However, it is unclear whether long-term
29 SS training program affects muscle stiffness or other viscoelastic properties of MTU
30 during passive stretching.

31 The purpose of this study was to determine the effect of a four-week SS training
32 program on the human gastrocnemius MTU, i.e. the properties of the muscle and other
33 structures *in vivo*.

34 Methods

35 Participants

36
37
38
39 Eighteen healthy males volunteered for this study (mean age: 21.4 years \pm 1.7, mean
40 height: 172.6 cm \pm 6.6, mean weight: 63.0 kg \pm 5.2). Subjects with a history of
41 neuromuscular disease or musculoskeletal injury involving their lower limbs were
42 excluded. This study was approved by the ethics committee of the Kyoto University
43 Graduate School and by the Faculty of Medicine (E-816), and it conformed to the
44 principles set out in the Declaration of Helsinki.

45 After successfully completing the initial test session, participants were randomly
46 assigned to either a stretching group (N = 9) or a control group (N = 9). The

1 characteristics of the subjects and the ROM of dorsiflexion for each group are presented
2 in Table 1. Unpaired t-test results indicated no significant differences for these
3 characteristics between the 2 groups.

4 5 SS training program

6
7 The participants in the stretching group were required to hold the SS position for 60 s
8 and complete 2 repetitions, for a total of 120 s of stretch during each session. Each
9 session was performed on a daily basis over a 4-week period. The stretch manoeuvre
10 was self-administered by participants. The participants stood with arms supported on
11 the wall anterior to the body. Both legs were straight, the hip in neutral rotation, with
12 only the forefoot resting on the platform. The ankle joint was dorsiflexed progressively
13 by leaning towards the wall until they felt the largest stretch that they were willing to
14 tolerate. The participants filled out a form on a daily basis to register compliance. They
15 were instructed not to initiate any new form of training. They did not perform any
16 stretch training on the day of the test.

17 18 Measurements

19
20 All measurements were performed prior to the SS training program (PRE) and after the
21 SS training program (POST). The participants were familiarised with the procedure and
22 were instructed to remain relaxed during measurement. To take into account the
23 influence of the intervention, POST measurements in the stretching group were
24 performed at least 24 hours after the last SS session. The person taking the
25 measurements was not blinded to the intervention.

26 27 ROM of dorsiflexion

28
29 The participants were secured at the hip in a prone position on the dynamometer
30 (MYORET RZ-450; Kawasaki Heavy Industries, Kobe, Japan), with their knees in full
31 extension and the foot of their dominant leg attached securely to the footplate of the
32 dynamometer. The footplate of the dynamometer was moved at a constant velocity of 1
33 degree/s by the motor control of the dynamometer, starting from 0 degrees to the
34 dorsiflexion angle at which the participants felt discomfort or pain. This dorsiflexion
35 angle was defined as the ROM.

36 37 Passive torque

38
39 Passive plantar flexion torque was measured using the dynamometer; participants held a
40 position similar to that used during the measurement of ROM. The footplate of the
41 dynamometer was moved at a constant velocity of 1 degree/s from 0 to 30 degrees of
42 dorsiflexion, which was achieved by all participants without pain. Passive plantar
43 flexion torque was measured at 0 and 30 degrees during passive ankle dorsiflexion.

44 45 Ultrasound measurements

1 B-mode ultrasonography (Famio Cube SSA-520A; Toshiba Medical Systems
2 Corporation, Tochigi, Japan) was used to determine the displacement of MTJ of the
3 medial head of the gastrocnemius muscle (GM) during passive ankle dorsiflexion. MTJ
4 was identified as described Maganaris and Paul (1999) and visualised as a continuous
5 sagittal plane ultrasound image using an 8 MHz linear-array probe. An acoustically
6 reflective marker made from softened vinyl film, was placed between the skin and the
7 probe to confirm that the probe did not move during measurements (Morse et al. 2008).
8 We defined MTJ displacement as the distance between MTJ and the acoustically
9 reflective marker secured to the probe. A custom-made fixation device was used to
10 secure the probe to the skin. Ultrasound images of the MTJ were quantified using the
11 open source digital measurement software (Image J, NIH, USA). The MTJ was
12 identified at the inner-most edges of the fascia surrounding the muscle where it fuses
13 with the tendon, to accurately measure MTJ. MTJ displacement was measured between
14 0 and 30 degrees of ankle dorsiflexion (Fig. 1).

15 Another B-mode ultrasonography (LOGIQe; GE Healthcare Japan, Tokyo,
16 Japan) was used to obtain fascicle length, which was calculated from the muscle
17 thickness and pennation angle. GM thickness was measured halfway along the lower
18 leg; i.e. at a point equidistant from the lateral malleolus of the fibula and the lateral
19 condyle of the tibia. The pennation angle of GM was determined from the angle of
20 fascicle insertion into the deep aponeurosis. Muscle thickness and the pennation angle
21 were measured at 0 and 30 degrees of ankle dorsiflexion. Movement of dynamometer
22 was stopped at 0 and 30 degrees by the motor, and the ultrasonography and
23 dynamometer measurements were performed synchronously. The fascicle length was
24 calculated using the following formula (Kumagai et al. 2000):

$$25 \text{ Fascicle length} = \text{muscle thickness} / \sin(\text{pennation angle})$$

26
27 Morse et al. (2008) reported that the contribution of the fascicle to MTU length
28 is proportional to the cosine of the angle of pennation; i.e. the fascicle length resolved
29 along the axis of the muscle (resolved fascicle length). The pennation angle decreases
30 with ankle dorsiflexion while the fascicle length and resolved fascicle length increase.
31 The change in factors other than muscle fascicle length was estimated from the change
32 in the resolved fascicle length (Δ resolved fascicle length) and MTJ displacement (Δ
33 MTJ) from 0 to 30 degrees of ankle dorsiflexion, elaborated as follows (Morse et al.
34 2008):

35 Change in factors other than fascicle length = Δ MTJ at 30 degrees – Δ resolved fascicle
36 length (Fig. 2)

37 38 EMG

39
40 We monitored the electromyographic (EMG) activity in the GM during the test
41 procedure (for about 40 sec) to confirm that the subject was relaxed and to ensure the
42 absence of high EMG activity. EMG activity was recorded using an EMG system
43 (TeleMyo2400; Noraxon USA, Inc., Scottsdale, AZ, USA) with surface electrodes
44 (11mm; Blue Sensor N, Ambu, Denmark). EMG activity was calculated using the root
45 mean square (RMS), and full wave rectification was performed using a RMS smoothing
46 algorithm at a window interval of 50 ms. The EMG activity within 3 sec was calculated

1 during isometric maximum voluntary contraction (MVC) of the ankle plantar flexors
2 with the ankle kept at 0 deg. The EMG activity recorded during the tests was expressed
3 as a percentage of MVC. The EMG sampling rate was 1500 Hz.

4 Reliability of the ultrasound measurements of MTJ

6 The ultrasonographic measurement of the MTJ displacement from 0 deg to 30 deg was
7 repeated twice on different days to assess test-retest reliability (n=7). Tests were
8 performed with at least 1 week interval, but not longer than 2 weeks, between the two
9 tests.

11 Statistics

12 SPSS (version 17.0; SPSS Japan INC., Tokyo, Japan) was used for statistical analyses.

13 For all variables, significant differences between PRE and POST were
14 determined in both the stretching and control groups using a paired t-test. Significance
15 of differences between the stretching and control groups at PRE was assessed using an
16 unpaired t-test. Significant differences were determined between within-group changes,
17 defined as POST minus PRE, in both the stretching and control group using an unpaired
18 t-test. In addition, a two-way ANOVA [(groups) × (test time)] was used to analyse the
19 interaction effects. Differences were considered statistically significant at an alpha level
20 of $P < 0.05$.

21 The reliability of MTJ displacement measurements was examined using the
22 intraclass correlation coefficient (ICC). Descriptive data are shown as means ± S.E.M
23 and 95% confidence intervals (CI) for the differences in changes score.

25 Results

26 Reliability and validity of ultrasound measurements

27
28 ICC for MTJ displacement measurements was 0.985 (trial 1: 1.21 ± 0.08 cm, trial 2:
29 1.22 ± 0.09 cm). During all tests, the EMG activity of GM was <2% MVC, which
30 confirmed the lack of a contractile component contribution to the passive torque, MTJ
31 displacement, muscle thickness and pennation angle.

32 Change in the ROM

33
34
35 There was no significant difference in the ROM between the stretching and control
36 groups at PRE. The ROM increased significantly after SS training in the stretching
37 group, whereas no significant difference was noted between PRE and POST in the
38 control group (Table 2). A two-way ANOVA showed a significant interaction effect (F
39 = 10.9, $P < 0.01$).

40 Changes in the passive torque and MTJ displacement

41
42
43 There were no significant differences in the passive torque at 0 and 30 degrees
44 dorsiflexion between the stretching and control groups at PRE. The passive torque at 0
45
46

1 degrees showed no significant differences between PRE and POST in either the
2 stretching or the control group. However, the passive torque value at 30 degrees
3 decreased significantly after SS training, whereas no significant difference was noted
4 between PRE and POST in the control group (Table 2). A significant interaction effect
5 was observed ($F = 15.1, P < 0.01$).

6
7 There was no significant difference in MTJ displacement between the stretching and the
8 control groups at PRE. MTJ displacement increased significantly at 30 degrees
9 dorsiflexion after SS training, whereas no significant difference was noted between PRE
10 and POST in the control group. There was a significant interaction effect ($F = 43.6, P <$
11 0.01).

12 13 Change in the fascicle length and resolved fascicle length

14
15 Table 2 shows changes in the fascicle length and resolved fascicle length. The fascicle
16 length and resolved fascicle length at 0 and 30 degrees showed no significant
17 differences between PRE and POST in either stretching or control group. The fascicle
18 length at 0 and 30 degrees showed no significant interaction effects ($F = 0.09; P = 0.76,$
19 $F = 0.03$ and $P = 0.88$, respectively) and the resolved fascicle length at 0 and 30 degrees
20 also showed no significant interaction effects ($F = 0.10; P = 0.76$ and $F = 0.02; P = 0.89,$
21 respectively).

22 23 Change in factors other than muscle fascicle length

24
25 Regarding the change in resolved fascicle length (Δ resolved fascicle length) from 0 to
26 30 degrees of ankle dorsiflexion, no significant difference was observed between PRE
27 and POST in either stretching or control group (Table 2). No significant interaction
28 effect was observed ($F = 0.85, P = 0.37$).

29 With regard to the value for factors other than muscle fascicle length, calculated
30 by subtracting Δ resolved fascicle length from Δ MTJ from 0 to 30 degrees, a
31 significant increase was observed after SS training in the stretching group. However, no
32 significant difference was noted between PRE and POST in the control group. A
33 significant interaction effect was observed ($F = 84.4, P < 0.01$).

34 35 Discussion

36 37 Reliability and validity of ultrasound measurements

38
39 We assessed the test-retest reliability of our ultrasound measurement of MTJ
40 displacement using the ICC. The measured MTJ displacement value demonstrated
41 substantial agreement because the ICC score was 0.985.

42 During measurement, the EMG activity of GM was very low ($<2\%$). It thus
43 seems rational that no voluntary or reflex contraction occurred during measurement,
44 which indicates that the measured passive torque and MTJ displacement values reflect
45 passive properties of MTU.

1 The change in MTU after SS training program

2
3 In this study, the ROM increased significantly in the stretching group, although no
4 significant change was found in the control group. This result suggests that a four-week
5 SS training program is effective for increasing ROM, which is consistent with previous
6 studies (Chan et al. 2001; Covert et al. 2010; Gajdosik 1991, 2001; Marques et al. 2009;
7 Reid and McNair 2004; Santonja Medina et al. 2007). Animal studies showed that the
8 number of sarcomeres in a series of muscles can be changed by prolonged
9 immobilisation in extreme positions (Goldspin.G et al. 1974; Tabary et al. 1972;
10 Williams and Goldspink 1978), leading to speculation that increases of maximum ROM
11 may be related to increases in the number of sarcomeres in series, and a concurrent
12 increase in length of the stretched muscles (Chan et al. 2001; Gajdosik 1991, 2001; Reid
13 and McNair 2004). However, in our study, the fascicle length (calculated from muscle
14 thickness and pennation angle) did not change after SS training program, which
15 suggests that the muscle length was not increased by SS training in vivo.

16 Morse et al. (2008) investigated the immediate effects of five-minute SS
17 sessions on not only the flexibility of overall MTU but also on muscle and tendon
18 stiffness. However, the effects of a long-term SS training program on MTU properties
19 remain unclear. In this study, the passive torque at 30 degrees decreased significantly
20 from PRE to POST in the stretching group, whereas no significant changes were evident
21 over time in the control group. These results suggested that the SS training program
22 decreased the stiffness of the overall MTU. In addition, MTJ displacement increased
23 significantly in the stretching group, whereas no significant change was noted in the
24 control group. These results suggest that the stiffness of the gastrocnemius muscle
25 decreased after the four-week SS training program. Our findings show that the SS
26 training program is effective in decreasing MTU stiffness, in particular muscle stiffness,
27 which is consistent with the immediate effect of SS (Morse et al. 2008). In this study,
28 POST measurements in the stretching group were performed at least 24 hours after the
29 last SS session to exclude the influence of the intervention. Therefore, we consider that
30 these results depend not on the immediate effects of stretching, but on the long-term
31 effects of the four week intervention.

32 With regard to the mechanism of decrease in muscle stiffness, Morse et al.
33 (2008) reported that MTJ displacement and the change in resolved fascicle length were
34 virtually identical before passive stretching, whereas MTJ displacement was 0.19 cm
35 greater than the change in resolved fascicle length after 5 min of SS training. They
36 therefore concluded that the additional displacement of MTJ was, at least in part, the
37 result of changes in structures other than the muscle fascicle. In this study, MTJ
38 displacement in the stretching group PRE was almost completely explained by the
39 change in fascicle length from 0 to 30 degrees of ankle dorsiflexion, since the change in
40 factors other than muscle fascicle length before SS training was negligible (0.01 cm).
41 However, a significantly larger change in factors other than muscle fascicle length was
42 observed after SS training (PRE: 0.01 cm, POST: 0.24 cm). On the other hand, there
43 were no significant changes in fascicle length and resolved fascicle length after SS
44 training. This suggests that the increased MTJ displacement observed after SS training
45 might be associated with factors other than muscle fibre length; i.e. components of
46 MTU proximal to MTJ.

1 Muscle fibres are surrounded by a complex connective tissue network, which
2 includes the endomysium, perimysium and epimysium. Perimysium bundles contain
3 several muscle fibres, while the endomysium surrounds individual muscle fibres.
4 Furthermore, the epimysium covers the entire muscle. Gajdosik et al. (2001) suggested
5 that the cytoskeleton of the sarcomere and the intramuscular connective tissue are made
6 up of parallel elastic components; for example, the endomysium, perimysium and
7 epimysium; which cause passive tension and therefore modification of these tissues
8 could lead to a change in MTU stiffness. Furthermore, Purslow (1989) reported that the
9 connective tissue, particularly the perimysium, is a major extracellular contributor to
10 passive stiffness. Thus, changes in properties of the intramuscular connective tissue that
11 cause passive tension are considered to be related to a decrease in muscle stiffness. In
12 addition, Kubo et al. (2002) reported that three-week SS training program decreased
13 MTU stiffness but not tendon stiffness, which implied that SS training program affected
14 the connective tissue elements in parallel with the muscle fibres. Taken together,
15 changes in properties of the intramuscular connective tissue such as the endomysium,
16 perimysium and epimysium instead of lengthening of muscle fiber, may also contribute
17 to the decrease in muscle stiffness found in our study.

18 There are some limitations of this study. First, the person taking measurements
19 was not blinded to the intervention. Second, the MTJ of the medial head of the
20 gastrocnemius muscle is not sharply delineated. In performing the MTJ measurements,
21 we identified the MTJ at the inner-most edges of the fascia surrounding the muscle
22 where it joins the tendon. The test-retest reliability of the measurement of MTJ
23 displacement in this study was very high. Therefore, we consider that the error in
24 measuring MTJ displacement was negligible. The third limitation of this study is that
25 passive torque is influenced not only by the gastrocnemius muscle but also by joint
26 capsules, ligaments and other muscles such as the soleus muscle. Further work is
27 needed to clarify the effect of long-term SS training program focusing on these factors.

28 In conclusion, the results of this study suggests that a four-week SS training
29 program decreases passive torque and increases the MTJ displacement of the
30 gastrocnemius at 30 degrees dorsiflexion, without causing changes in muscle fascicle
31 length.

32 33 Conflict of Interest Statement

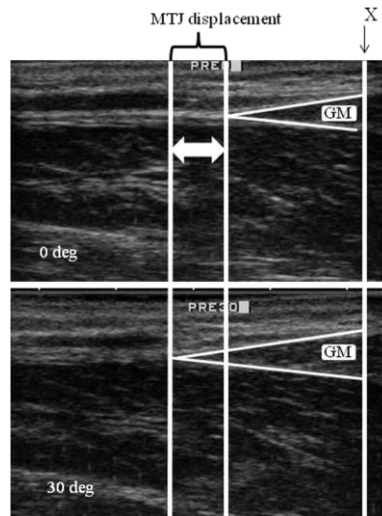
34
35 The authors have no conflicts of interest to declare.
36

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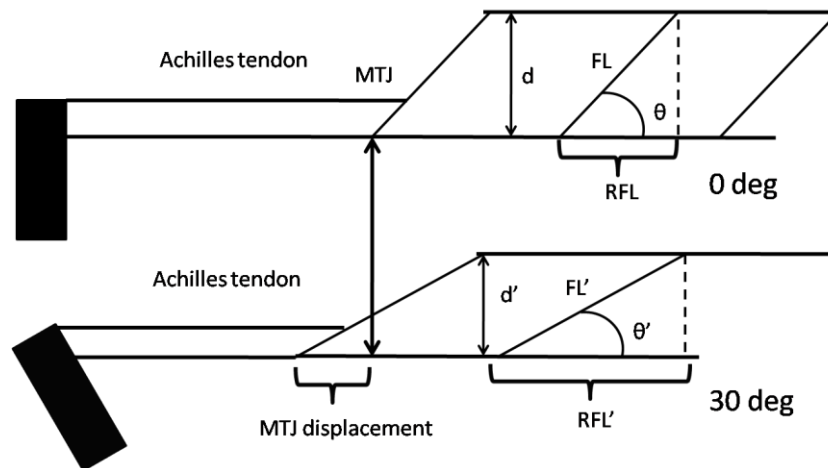
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35

1 Figure 1.
 2 Ultrasound imaging showing the measurements taken to determine MTJ displacement



3
 4 An acoustically reflective marker (X) was placed between the skin and the ultrasonic
 5 probe to confirm that the probe did not move during measurements. The distance
 6 between X and the myotendinous junction (MTJ) of the medial head of the
 7 gastrocnemius muscle (GM) was measured at 0 and 30 degrees, and the difference
 8 between 0 and 30 degrees was defined as “MTJ displacement”.

9
 10 Figure 2.
 11 Schematic diagram showing measurements taken to determine fascicle length, resolved
 12 fascicle length and changes in factors other than muscle fascicle length



13
 14 d: muscle thickness, θ : pennation angle, FL: fascicle length, RFL: resolved fascicle
 15 length, MTJ: myotendinous junction
 16 $FL = d/\sin\theta$, $RFL = FL \times \cos\theta$
 17 Changes in factors other than fascicle length = MTJ displacement - (RFL' - RFL)
 18

1 **Table 1.**

2 **Characteristics of subjects in the stretching group and those in the control group.**

	stretching group (N=9)	control group (N=9)
age (years)	21.1±2.3 (19-26)	21.8±0.8 (20-23)
height (cm)	173.8±6.8 (163-184)	171.4±6.6 (165-186)
weight (kg)	64.4±4.9 (54-70)	61.6±5.4 (53-72)
ROM (deg) ^a	32.9±2.8 (30-36)	32.0±3.0 (30-37)

3 Data are means ± S.E.M. (95% confidence interval)

4 ^aROM=Range of Motion

6 **Table 2.**

7 **Changes in variables between PRE and POST in both the stretching and control**
 8 **groups.**

	Stretching Group			Control Group			P-value of change ^h	Interaction effect ⁱ
	PRE	Change ^f	95%CI ^g	PRE	Change ^f	95%CI ^g		
ROM (deg) ^a	32.9±2.8	6.7±1.7**	2.8 to 10.5	32.0±3.0	-0.7±0.7	-2.2 to 0.9	P < 0.01	P < 0.01
Passive torque at 0deg (Nm)	3.6±0.6	1.0±0.7	-0.6 to 2.5	4.0±0.4	0.0±0.3	-0.8 to 0.8	P=0.21	P = 0.21
at 30 deg (Nm)	46.2±5.6	-6.2±1.1**	-8.8 to -3.6	37.4±2.8	2.2±1.8	-2.0 to 6.4	P < 0.01	P < 0.01
MTJ displacement at 30 deg (cm) ^b	0.93±0.06	0.40±0.06**	0.27 to 0.53	1.03±0.09	0.01±0.01	-0.02 to 0.04	P < 0.01	P < 0.01
fascicle length at 0 deg (cm)	5.9±0.5	-0.3±0.4	-1.3 to 0.8	5.8±0.3	0.0±0.1	-0.2 to 0.2	P=0.60	P = 0.76
at 30 deg (cm)	6.8±0.5	-0.1±0.4	-1.2 to 1.0	6.7±0.3	0.0±0.0	0.0 to 0.1	P=0.79	P = 0.88
difference of fascicle length (cm) ^c	0.89±0.05	0.15±0.04	-0.04 to 0.33	0.98±0.08	0.03±0.01	-0.22 to 0.28	P=0.38	P = 0.43
resolved fascicle length at 0 deg (cm)	5.7±0.5	-0.3±0.4	-1.3 to 0.8	5.5±0.3	0.1±0.3	-0.1 to 0.2	P=0.60	P = 0.76
at 30 deg (cm)	6.6±0.5	-0.1±0.4	-1.2 to 0.4	6.6±0.3	0.0±0.3	0.0 to 0.1	P=0.81	P = 0.89
Δ resolved fascicle length (cm) ^d	0.92±0.06	0.17±0.07	-0.02 to 0.36	1.00±0.08	0.03±0.02	-0.01 to 0.08	P=0.14	P = 0.37
factors other than fascicle length (cm) ^e	0.01±0.01	0.22±0.02**	0.17 to 0.27	0.03±0.01	-0.01±0.01	-0.05 to 0.02	P < 0.01	P < 0.01

10 **: P < 0.01 significant difference in change between PRE and POST.

11 Data are means ± S.E.M.

12 ^aROM = Range of Motion

13 ^bMTJ = Myotendinous junction displacement at 30 deg

14 ^c difference of fascicle length = fascicle length at 30 deg – fascicle length at 0 deg

15 ^dΔ resolved fascicle length = resolved fascicle length at 30 deg – resolved fascicle
 16 length at 0 deg

17 ^e factors other than fascicle length = MTJ displacement – Δ resolved fascicle length

18 ^fChange = POST value – PRE value

19 ^g 95 % CI: 95 % confidence intervals

20 ^h Difference of change was determined in both stretching and control group using an
 21 unpaired t-test.

22 ⁱ Interaction effect was determined using a two-way ANOVA [(groups) × (test time)].