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ORIGINAL ARTICLE

Low-Level Laser Therapy Prevents Treadmill Exercise-Induced Progression of Arthrogenic Joint Contracture *Via* Attenuation of Inflammation and Fibrosis in Remobilized Rat Knees

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Abstract—We investigated whether the combination of exercise and anti-inflammatory/anti-fibrotic treatment using low-level laser therapy (LLLT) promotes recovery from joint contracture without arthrogenic contracture progression. Rat knees were immobilized for 3 weeks in a flexed position. After fixator removal, rats were divided into no intervention (RM), daily treadmill walking (WALK), and daily treadmill walking and LLLT (W + L) groups. Total and arthrogenic contractures were assessed by restrictions of passive range of motion (ROM) before (m-ROM) and after myotomy (a-ROM), respectively. After 7 days of remobilization, m-ROM restriction decreased equally in all groups. Conversely, a-ROM restriction further increased after remobilization in the RM and WALK groups. Furthermore, this restriction was significantly larger in the WALK group compared with the RM group. In the W + L group, however, progression of a-ROM restriction during remobilization was prevented. After 1 or 7 days of remobilization, inflammatory and fibrotic reactions in the joint capsule were induced in the RM group and were more pronounced in the WALK group, but these reactions were milder in the W + L group than in the WALK group. m-ROM restriction representing total contracture initially established by immobilization was partially improved by remobilization. Additional LLLT and exercise intervention did not further reduce total contracture, but LLLT suppressed the progression of arthrogenic contracture caused by ambulation and treadmill exercise. Therefore, exercise with LLLT in the early phase of remobilization would be one possible adjunct therapy to prevent further progression of arthrogenic contracture.

KEY WORDS: exercise; fibrosis; remobilization; inflammation; joint contracture; low-level laser therapy.

INTRODUCTION

Joint immobilization is used to maintain joint stability following injury of periarticular structures, such as bone, tendon, and ligament, but simultaneously causes joint contractures [1, 2]. Joint contractures require prompt recovery because they can limit activity [3, 4]. To date, facilitating joint movement is important for improving joint contractures. In fact, immobilization-induced joint contracture is clinically treated by physical therapy during remobilization [2, 5]. Several animal studies have also reported that treadmill exercise during remobilization promotes recovery

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from joint contracture [6, 7]. However, other studies show adverse effects of active exercise on joint contracture. Two independent structures, muscle and articular components, contribute to the formation of joint contracture and each respond to immobilization and remobilization differently [8–12]. Thus, we divided joint contracture to two categories, namely muscular and articular-origins. Remobilization after 3 weeks of immobilization partially recovered overall joint range of motion (ROM) by improving muscular-origin contracture while aggravating arthrogenic contracture (articular-origin) through inflammation and subsequent fibrosis in the joint capsule [9, 13, 14]. Another study also showed that remobilization following short-term (1 or 2 weeks) immobilization improves muscular-origin contracture, but develops arthrogenic contracture [12]. Moreover, treadmill exercise during remobilization promotes the progression of arthrogenic contracture through enhanced inflammatory and fibrotic reactions in the joint capsule [13]. Taken together, these studies suggest that active joint movement after immobilization can improve overall ROM by improving muscular-origin contracture, while further developing arthrogenic contracture. Since it is more difficult to recover from arthrogenic than muscular-origin contracture [12, 15], preventing the progression of arthrogenic contracture during remobilization is important for improving joint contracture therapy.

Previously, we have revealed that anti-inflammatory treatments during remobilization, such as low-level laser therapy (LLLT) and administration of dexamethasone (steroidal anti-inflammatory drug), prevent arthrogenic contracture progression with accompanying fibrotic reactions in the joint capsule [14, 16]. Thus, we consider that the combination of treadmill walking and anti-inflammatory/anti-fibrotic treatment using LLLT during remobilization promotes recovery from immobilization-induced joint contracture without progression of arthrogenic contracture. However, the effects of combining exercise and LLLT on joint contracture have not been examined. To test our hypothesis, we investigated the effects of combining treadmill walking and LLLT on ROM and histopathology, as well as gene expression of inflammatory and fibrotic mediators in remobilized rat knees.

MATERIALS AND METHODS

Experimental Animals

Fifty-eight male Wistar rats (8-week-old, 180–230 g; Japan SLC, Shizuoka, Japan) were used in this study. The rats were randomly divided into immobilization (IM: $n=9$), remobilization without intervention (RM: $n=17$), remobilization with daily treadmill walking (WALK: $n=16$), and remobilization with daily treadmill walking and LLLT (W + L: $n=16$) groups. Untreated left knees in the IM group were used as controls. Some rats in the RM and WALK groups were used as part of our previous study [13]. Rats were housed in standard cages in a temperature-controlled room (20–25 °C) with 12 h light/dark cycles. Standard rodent chow and water were provided *ad libitum*. This experimental design was approved by the committee on animal experimentation of Hiroshima International University.

Joint Immobilization and Remobilization

The right knees were immobilized as described previously [8, 10]. In brief, after anesthesia by intraperitoneal injection of sodium pentobarbital (32.4 mg/kg of body weight), Kirschner wires (01-132-50; MIZUHO, Tokyo, Japan) were screwed into the femur and tibia. We then fixed knees to 140° using Kirschner wires with wire and resin (PROVINICE FAST; Matsukaze, Kyoto, Japan). After 3 weeks, the fixation device was removed (remobilization). In the IM group, data were collected immediately after removing the fixator to assess the effects of immobilization without remobilization.

Treadmill Walking and LLLT

In RM, WALK, and W + L groups, rats were allowed to recover after removing the fixator. During remobilization, rats in the RM group were allowed to move freely in a standard cage without any intervention. In the WALK and W + L groups, rats performed daily treadmill walking on a motor-driven treadmill machine (Rat runner; Agawa, Shimane, Japan) at 12 m/min for 60 min/day [13]. The walking intervention consisted of six 10 min walking sessions separated by 1 min intervals. In the W + L group, rats were anesthetized by inhalation of diethyl ether immediately after treadmill walking, after which LLLT was applied to the right knee using semiconductor laser systems (FINE LASER EL-800, Panasonic Healthcare, Tokyo, Japan). Laser irradiation was performed using the skin contact method at the medial and lateral sides of the knee under the following conditions: continuous irradiation mode, wave length 830 nm, power output 150 mW, power density 5 W/cm^2, and irradiation time 60 s/point. This irradiation condition can attenuate remobilization-induced fibrotic reactions in the joint capsule [16]. In RM, WALK, and W + L groups, rats were sacrificed after 1 day ($n = 8$) or 7 days ($n = 8$ or 9) of remobilization and data were collected.

Measurement of ROM

Passive knee joint ROMs were measured according to methods from our previous study [17]. Rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (32.4 mg/kg of body weight) and inhalation of diethyl ether when required. After removing skin from the hind limbs, rats were placed in the neutral spine position and the trunk and femur were manually fixed at 90° hip flexion. We then applied 14.6 N·mm of knee extension moment, which extends the knee close to its physiological limit [18], but does not disrupt the knee soft tissue [11], to the lower leg using a pulley and weight. The angle between the femur and fibula was measured as an m-ROM knee extension using a 3D motion analysis system (Kinema Tracer; KISSEI COMTEC, Nagano, Japan).

To assess arthrogenic factor, knee flexor muscles were completely transected after sacrifice by exsanguination. We then re-measured the ROM extension as an a-ROM. Based on the m-ROM and a-ROM values, the degrees of total and arthrogenic contractures were calculated according to the method described previously [11]. The formulae used were as follows:

(1) total contracture = ROM before myotomy (of the control knee) − ROM before myotomy (of the contractured knee); (2) arthrogenic contracture = ROM after myotomy (of the control knee) − ROM after myotomy (of the contractured knee).

Histological Assessment

Tissue Preparation

After ROM measurements, knees were harvested and immersion-fixed with 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4) for 2 days at 4 °C. During fixation, knees were kept at 90° flexion. We then decalcified samples in 17.7% ethylenediaminetetraacetic acid (pH 7.2, OSTEOSOFT; Merck Millipore, Darmstadt, Germany) for 4 weeks at room temperature, after which samples were embedded in paraffin. Four micrometers sagittal sections were obtained at the medial mid condylar level and were stained with either aldehyde fuchsin-Masson Goldner (AFMG) or hematoxylin and eosin (HE).

Morphometric Analysis of Joint Capsule

Using AFMG-stained sections, the postero-superior and postero-inferior synovial lengths of the posterior joint capsule were measured according to the method described by Trudel et al. [19] (Supplemental Fig. 1a). To calculate the total synovial length, we summed the postero-superior and postero-inferior synovial lengths.

We also measured the thickness of the posterior joint capsule as previously described [13] (Supplemental Fig. 1a). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure synovial length and joint capsule thickness.

Calculation of Joint Capsule Collagen Density

Using ImageJ, the collagen density of the posterior joint capsule was measured according to methods from our previous study [9] (see details in Supplemental Fig. 1b–d).

Counting of Cells

The superior, central, and other regions of the posterior joint capsule of the HE-stained sections were photographed under ×40 magnification. We manually counted the number of hematoxylin-stained cell nuclei and cell number was represented as cells per square millimeter of joint capsule area.

Counting of Fibroblasts

To visualize fibroblasts, immunohistochemistry was performed for vimentin. After deparaffinization and rehydration, the sections were incubated with 1% trypsin for 5 min at 37 °C for antigen retrieval. Endogenous peroxidase activity was blocked by incubating with methanol containing 3% H_2O_2 for 30 min. After blocking of nonspecific binding for 30 min with 1% normal horse serum in 0.01 M phosphate-buffered saline (PBS; pH 7.4), the sections were incubated with an anti-vimentin antibody (1:1500 dilution; ab92547, Abcam, Cambridge, UK) overnight at 4 °C, followed by rinsing with PBS. We then added the secondary antibody (horse biotinylated anti-mouse/rabbit IgG, 1:250 dilution; BA-1400, Vector Laboratories, Burlingame, CA, USA) for 30 min. After rinsing with PBS, sections were incubated with a streptavidin-biotin complex (1:50 dilution; Elite ABC, Vector Laboratories, Burlingame, CA, USA) for 30 min. We then added Dako EnVision + kit/HRP (DAB) (Dako Japan, Tokyo, Japan) for color development. Finally, we counterstained sections with hematoxylin. Although vimentin is often used as a fibroblast marker [20, 21], it is also expressed in other cell types, including endothelial cells, macrophages, neutrophils, and lymphocytes [22]. Therefore, spindle-shaped vimentin-positive cells not detected in luminal structures were counted as fibroblasts [13]. The counting was performed in the same way as cell counting.

Immunohistochemistry for Type I and III Collagens

To assess joint capsule fibrosis, we performed immunohistochemistry for type I and III collagens, except for the samples obtained after 1 day of remobilization; this was due to observed increase in collagen density and upregulations of collagen genes after day 7, but not after day 1 of remobilization. Sections were deparaffinized and rehydrated with a descending alcohol series and were treated with proteinase K (0.05 mol/L; Dako Japan, Tokyo, Japan) for 5 min at room temperature. After rinsing with PBS, sections were incubated with methanol containing 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Sections were incubated with a blocking solution of PBS containing 1% normal horse serum for 20 min to eliminate nonspecific binding. We then incubated sections with either an anti-type I collagen antibody (C2456, 1:2000 dilution; Sigma-Aldrich, St. Louis, MI, USA) or an anti-type III collagen antibody (C7805, 1:8000 dilution; Sigma-Aldrich, St. Louis, MI, USA) overnight at 4 °C. The negative control was incubated with vehicle (1% bovine serum albumin). After rinsing with PBS, the secondary antibody (horse biotinylated anti-mouse IgG, 1:250 dilution; BA-2001, Vector Laboratories, Burlingame, CA, USA) was added for 30 min. Sections were rinsed with PBS and incubated with a streptavidin-biotin complex (1:50 dilution; Elite ABC) for 30 min. Finally, immunoreactivity was visualized with Dako EnVision + kit/HRP (DAB).

Gene Expression Analysis of Inflammation and Fibrosis-Related Factors

Total RNA in the posterior joint capsule was extracted from paraffin sections according to methods from our previous study [9]. In brief, sagittal sections were cut from the lateral side of the knee, after which we isolated the posterior joint capsule. Using an RNeasy FFPE Kit (Qiagen, Hilden, Germany), we extracted total RNA, then prepared cDNA using total RNA and the SuperScript III First-strand synthesis system (Invitrogen, Grand Island, NY, USA). cDNA samples were stored at − 20 °C until used for real-time PCR analyses.

Using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, US), we performed TaqMan probe-based real-time PCR assays. TaqMan primer and probe sets for interleukin-1β (*IL-1β*; Rn00580432_m1), transforming growth factor-β1 (*TGF-β1*; Rn00572010_m1), type I collagen (*COL1A1*; Rn01463848_m1), type III collagen (*COL3A1*; Rn01437681_m1), and *S18* (Rn01428913_gH) were designed and synthesized by Applied Biosystems. *S18* rRNA was used as an internal control. Relative quantifications of gene expression were carried out using the calibration curve method.

Statistical Analysis

All data were expressed as mean ± standard deviation. We performed statistical analyses using Dr. SPSS II for Windows (SPSS Japan Inc., Tokyo, Japan). Levene's test was used to test homogeneity of variance and we applied a one-way analysis of variance (ANOVA) to statistically evaluate the differences among groups. Regarding gene expressions, a data set that did not meet homoscedasticity assumptions, we applied the Games-Howell test to clarify the differences between the control or IM groups and other groups. Alternatively, we applied the Tukey's post-hoc test. We used two-way ANOVA to examine the relationship between intervention and time. For significant direct or interaction effects, we performed post-hoc Bonferroni tests to localize the effects. For all tests, a P value less than 0.05 was considered statistically significant.

RESULTS

Total and Arthrogenic Contractures

Total contracture represented by reduction in m-ROM in the IM group was 65 ± 6° (Table 1, Fig. 1a). One-day remobilization significantly reduced total contractures by restricting m-ROMs in the three groups on day 1 to a lesser extent than in the IM group ($P < 0.03$). Seven days of remobilization further reduced total contractures compared with those for 1-day remobilization ($P < 0.001$). Among the three groups, there were no differences in total contracture after either 1 or 7 days of remobilization ($P > 0.43$).

Knee extension ROMs were increased by myotomy in all groups, by at least 6° for the control group and a maximum of 48° for the IM group (Table 1). Arthrogenic contracture represented by reduction in a-ROM in IM group was 23 ± 6° (Table 1, Fig. 1b). After 1 day of remobilization, arthrogenic contractures did not differ between the three remobilization groups and the IM group ($P > 0.89$). After 7 days of remobilization, arthrogenic

Table 1. ROMs Before (m-ROM) and After Myotomy (a-ROM) in Various Stages of Artificially Immobilized and Remobilized Rat Knees

		m-ROM (deg)	a-ROM (deg)	Increase after myotomy (deg)
Control		155 ± 6	162 ± 5	6 ± 8
IM		90 ± 6	139 ± 6	48 ± 6
Day 1	RM	98 ± 5	137 ± 5	40 ± 9
	WALK	101 ± 4	136 ± 5	35 ± 7
	W + L	99 ± 3	136 ± 3	37 ± 4
Day 7	RM	109 ± 5	124 ± 6	16 ± 6
	WALK	109 ± 4	116 ± 6	7 ± 4
	W + L	108 ± 5	136 ± 4	28 ± 5

Values are mean ± standard deviation

contractures in both RM and WALK groups were significantly greater than in the IM group ($P < 0.001$). Between the RM and WALK groups, arthrogenic contracture was significantly greater in the WALK group ($P = 0.003$). In the W + L group, arthrogenic contracture was significantly milder than those in RM and WALK groups ($P < 0.001$); this also did not differ from the IM group ($P = 0.91$).

Histological Changes

The postero-superior joint capsule in the control group (Fig. 2a) showed deep synovial folds. In the IM group (Fig. 2b), synovial folds in the postero-superior joint capsule were shallower than in the control group. After 1 day of remobilization, we detected deposits in the posterior joint space in all three groups (Fig. 2c–e). After 7 days of remobilization, deposits almost disappeared and synovial folds in the postero-superior joint capsule were obstructed by fibrous tissues in all three groups (Fig. 2f–h).

The postero-central joint capsule in the control (Fig. 2a) and IM (Fig. 2b) groups was thin. After 1 day of remobilization, the postero-central joint capsule was thickened by edema in all three groups (Fig. 2c–e). After 7 days of remobilization, the joint capsule edema disappeared in all three groups (Fig. 2f–h). Postero-central joint capsule thickness in the RM group (Fig. 2f) returned to a level close to that in the control and IM groups; however, those in WALK (Fig. 2g) and W + L (Fig. 2h) groups were still thicker than in the control and IM groups.

Collagen bundles in the postero-central joint capsule in the control (Fig. 3a) and IM (Fig. 3b) groups were arranged with narrow gaps. After 1 day of remobilization, the gaps between collagen bundles were spread by edema in all three groups (Fig. 3c–e). After 7 days of remobilization, joint capsule edema was alleviated in all three groups compared to 1 day of remobilization in each group (Fig. 3f–h). The gaps between collagen bundles narrowed in all three groups, which was most prominent in the WALK group (Fig. 3f–h).

Synovial Length

Total synovial length in the IM group decreased significantly compared with the control group ($P < 0.001$; Fig. 2i). After 1 day of remobilization, synovial lengths in all three groups were comparable to that of the IM group ($P > 0.97$). After 7 days of remobilization, synovial lengths in all three groups were significantly shorter compared with the IM group ($P < 0.03$). Among the three groups, total synovial length did not differ irrespective of remobilization duration for either 1 or 7 days ($P > 0.40$).

Joint Capsule Thickness

There was no difference in joint capsule thickness between the control and IM groups ($P = 0.65$; Fig. 2j). After 1 day of remobilization, joint capsule thickness in all three groups was significantly thicker than those in the control and IM groups ($P < 0.001$). Compared with the RM group, joint capsule thickness in the W + L group was significantly thicker ($P = 0.047$). After 7 days of remobilization, joint capsule thickness in the RM group did not differ from those in the control and IM groups ($P > 0.05$). In contrast, joint capsule thickness in the WALK and W + L groups was still significantly thicker than those in the control and IM groups ($P < 0.01$). Between the RM and WALK groups, the joint capsule was significantly thicker in the WALK group ($P = 0.01$).

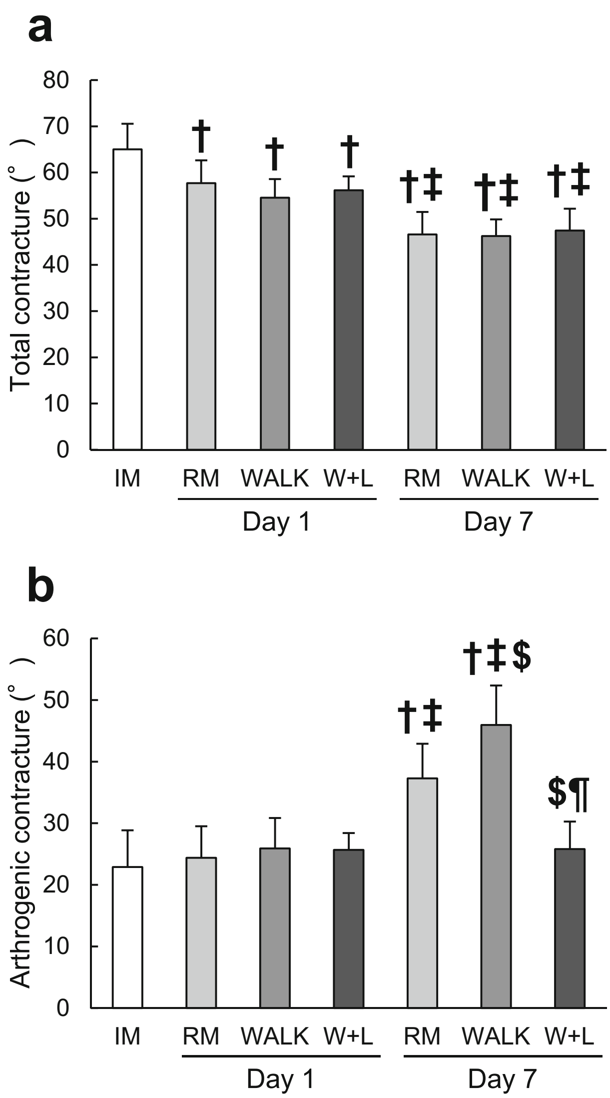

Fig. 1. Degrees of contracture. **a** Total contracture was partially recovered and comparable among all three groups at both time points (day 1 and 7) of remobilization. **b** Arthrogenic contracture was further developed at day 7 in both RM and WALK groups compared to each group at day 1 and the IM group. Between the RM and WALK groups at day 7, arthrogenic contracture was larger in the WALK group. In the W + L group, the development of arthrogenic contracture during remobilization was completely prevented. Values are mean ± standard deviation. †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$). ¶, significant difference compared with the WALK group at the same time point ($P < 0.05$).

Collagen Density

There was no difference in collagen density between the control and IM groups ($P = 0.99$; Fig. 3i). After 1 day of remobilization, collagen density decreased in all three groups ($P < 0.002$ compared with the control and IM groups). There were no differences among the three groups ($P = 1.00$). After 7 days of remobilization, collagen densities in all three groups were significantly higher than those in the control and IM groups ($P < 0.002$). Among the three groups, collagen density in the WALK group was significantly higher than those in the RM and W + L groups ($P < 0.02$).

Expression of Type I and III Collagens in the Posterior Joint Capsule

The staining of type I collagen in the posterior joint capsule in the control (Fig. 4a) and IM group (Fig. 4b) was sparse, and there was no apparent difference between the two groups. After 7 days of remobilization, the staining of type I collagen in the three groups (Fig. 4c–e) was denser than those in the control and IM groups. Among the three groups, the staining density of type I collagen was highest in the WALK group (Fig. 4d).

Likewise, there was no apparent difference in the staining of type III collagen in the posterior joint capsule between the control (Fig. 4f) and IM (Fig. 4g) groups. After 7 days of remobilization, the expression of type III collagen in all three groups (Fig. 4h–j) was substantially higher than those in the control and IM groups. Among the three groups, the staining density of type III collagen in the WALK group was the highest (Fig. 4i). In the negative control, we did not detect a positive signal (data not shown).

Cellularity

In the control (Fig. 5a) and IM (Fig. 5b) groups, spindle-shaped fibroblast-like cells and vascular endothelial cells were mainly observed in the posterior joint capsule, and inflammatory cells were rarely seen. There was no difference in cell number between the control and IM groups ($P = 1.00$; Fig. 5i). After 1 day of remobilization, circular inflammatory cell infiltration was observed in all three groups (Fig. 5c–e). However, cell numbers in all three groups were not significantly different from those in the control and IM groups ($P > 0.05$). We detected no significant differences among the three groups ($P > 0.27$). After

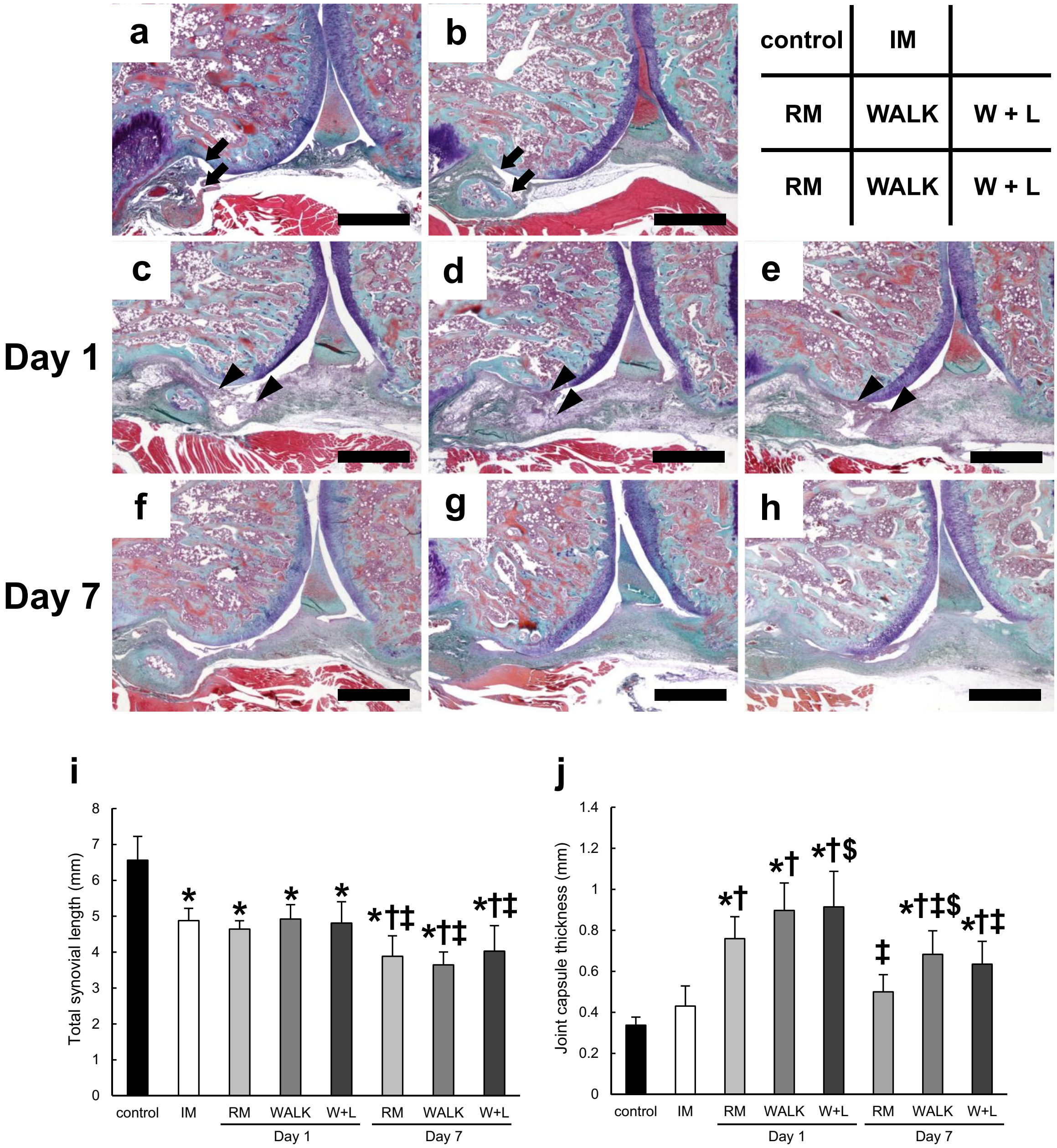

Fig. 2. Histomorphometric changes in the posterior knee joint capsule. Representative images of the AFMG-stained posterior knee joint for the **a** control, **b** IM, **c** RM on day 1, **d** WALK on day 1, **e** W + L on day 1, **f** RM on day 7, **g** WALK on day 7, and **h** W + L on day 7. **i, j** Total synovial length and thickness in the posterior joint capsule, respectively. Synovial folds (arrows) in the postero-superior joint capsule in the IM group (**b**) were shallower than the control group (**a**). Consequently, total synovial length in the IM group was significantly shortened compared with the control group (**i**). After 1 day of remobilization, we detected deposits (arrowheads) in the joint space, but synovial length was maintained at the IM group level in all three groups (**c**–**e**, **i**). After 7 days of remobilization, synovial folds disappeared and total synovial length was further shortened in all three groups (**f**–**i**). Thickness of the posterior joint capsule increased due to edema in all three groups after 1 day of remobilization (**c**–**e**, **j**). After 7 days of remobilization, posterior joint capsule thickness in the RM group (**f**), but not WALK (**g**) and W + L (**h**) groups, returned to a level close to that of the control and IM groups (**j**). Thickness of the posterior joint capsule in the WALK group was significantly thicker than in the RM group. Scale bars = 1 mm. Values are mean ± standard deviation. *, significant difference compared with the control group ($P < 0.05$). †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$).

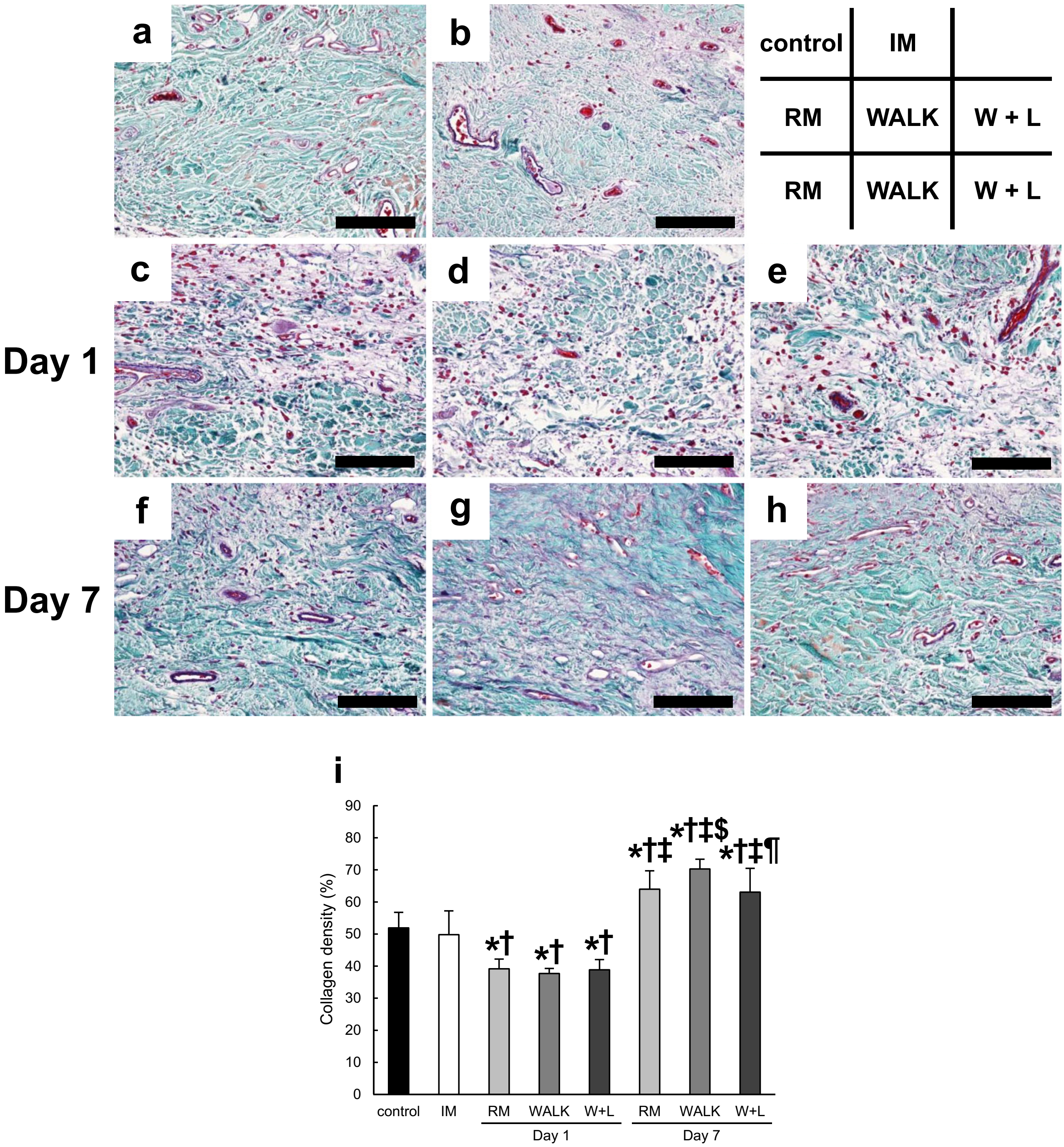

Fig. 3. Collagen density in the posterior joint capsule. Representative images of the AFMG-stained postero-central joint capsule in the **a** control, **b** IM, **c** RM on day 1, **d** WALK on day 1, **e** W + L on day 1, **f** RM on day 7, **g** WALK on day 7, and **h** W + L on day 7. The green color indicates collagen. **i** Collagen density. Compared with control (**a**) and IM groups (**b**), collagen density temporarily decreased due to edema in all three groups after 1 day of remobilization (**c**–**e**, **i**). After 7 days of remobilization, gaps between collagen bundles narrowed and collagen density increased in all three groups (**f**–**i**). Among the three groups, collagen density in the WALK group (**g**) was significantly higher than in the RM (**f**) and W + L groups (**h**). Scale bars = 100 µm. Values are mean ± standard deviation. *, significant difference compared with the control group ($P < 0.05$). †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$). ¶, significant difference compared with the WALK group at the same time point ($P < 0.05$).

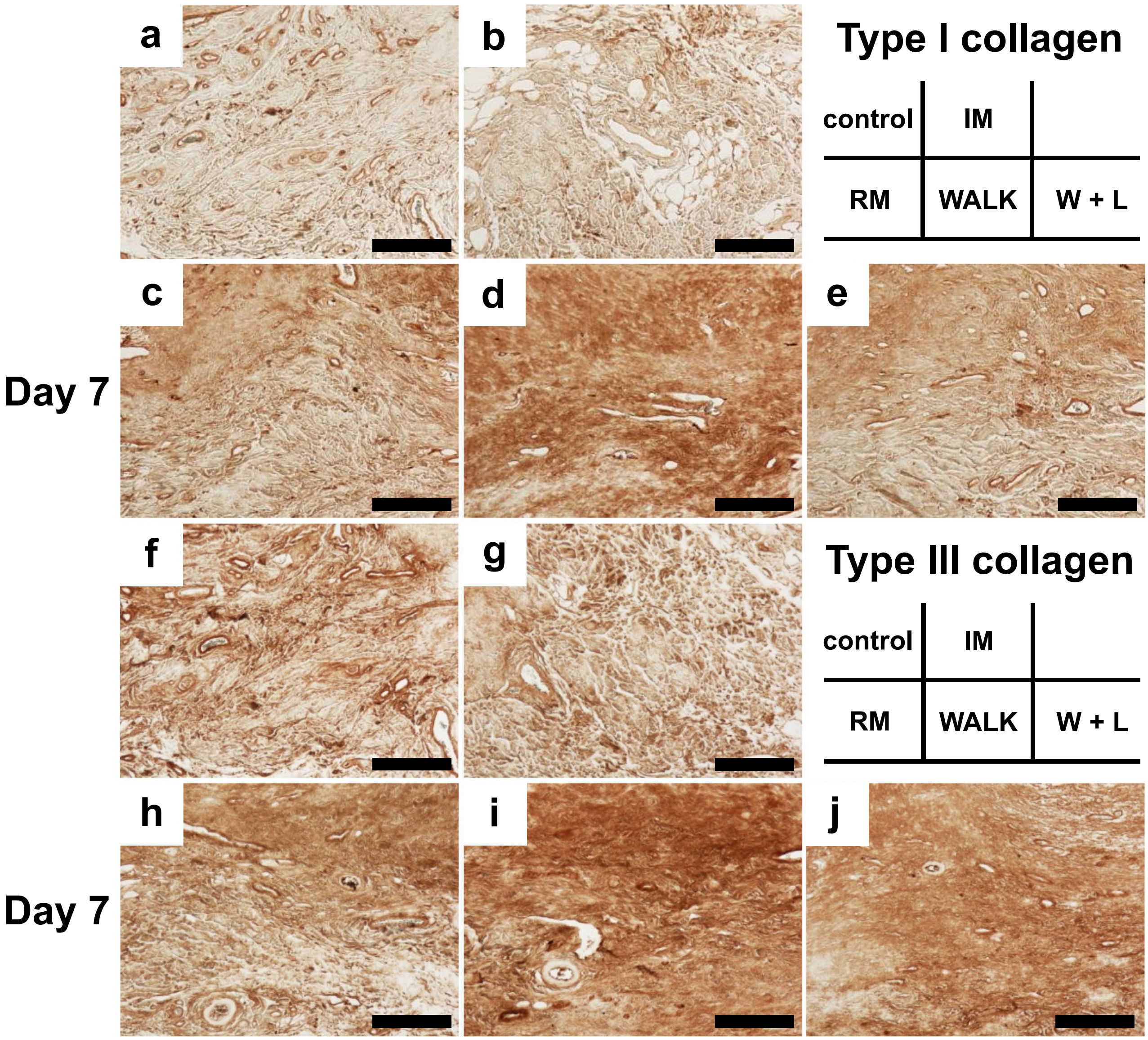

Fig. 4. Expression of type I and III collagens in the posterior joint capsule. **a**–**e** The expression of type I collagen in the postero-central joint capsule. **f**–**j** The expression of type III collagen in the postero-central joint capsule. **a**, **f** Control; **b**, **g** IM; **c**, **h** RM on day 7; **d**, **i** WALK on day 7; **e**, **i** W + L on day 7. Compared with the control and IM groups, the staining of both type I and III collagens was denser in all groups after 7 days of remobilization. Among the remobilized groups, the staining densities of both type I and III collagens were highest in the WALK group. Scale bars = 100 μm.

7 days of remobilization, the inflammatory cells had disappeared and spindle-shaped fibroblast-like cells became predominant in all three groups (Fig. 5f–h). Cell numbers in all three groups were significantly higher than those in the control and IM groups ($P < 0.001$). Among the three groups, cell numbers in the WALK group (Fig. 5g) were significantly higher than those in the RM (Fig. 5f) and W + L (Fig. 5h) groups ($P < 0.04$).

There was no difference in fibroblast number between the control (Fig. 6a) and IM (Fig. 6b) groups ($P = 0.77$; Fig. 6i). After 1 day of remobilization, the number of vimentin-positive fibroblasts in all three groups remained unchanged compared with the control and IM groups ($P > 0.70$, Fig. 6c–e). After 7 days of remobilization, vimentin-positive fibroblast numbers in all three groups were significantly higher than in the control and IM groups ($P < 0.001$; Fig.

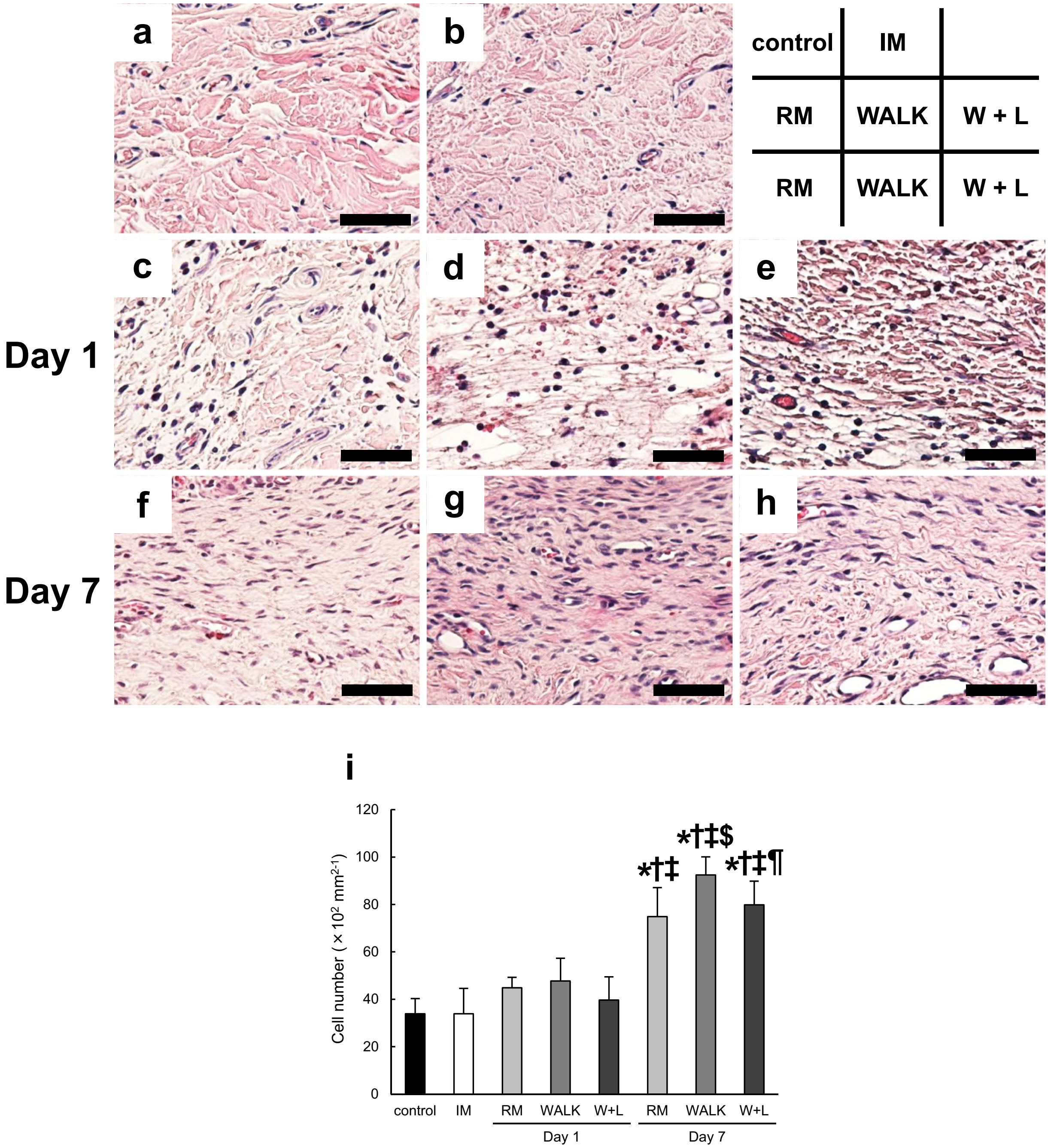

Fig. 5. Cellularity in the posterior joint capsule. Representative images of the HE-stained postero-central joint capsule in the **a** control, **b** IM, **c** RM on day 1, **d** WALK on day 1, **e** W + L on day 1, **f** RM on day 7, **g** WALK on day 7, and **h** W + L on day 7. **i** Cell number. In the control (**a**) and IM groups (**b**), the joint capsule mainly contained spindle-shaped fibroblast-like cells and vascular endothelial cells. After 1 day of remobilization, we also observed circular inflammatory cell infiltration, but cell number did not differ from the control and IM groups in all three groups (**c**–**e**, **i**). After 7 days of remobilization, cell number in all three groups significantly increased by proliferation of spindle-shaped fibroblast-like cells (**f**–**i**). Among the three groups, cell number in the WALK group (**g**) was significantly higher than in the RM (**f**) and W + L groups (**h**, **i**). Scale bars = 50 µm. Values are mean ± standard deviation. *, significant difference compared with the control group ($P < 0.05$). †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$). ¶, significant difference compared with the WALK group at the same time point ($P < 0.05$).

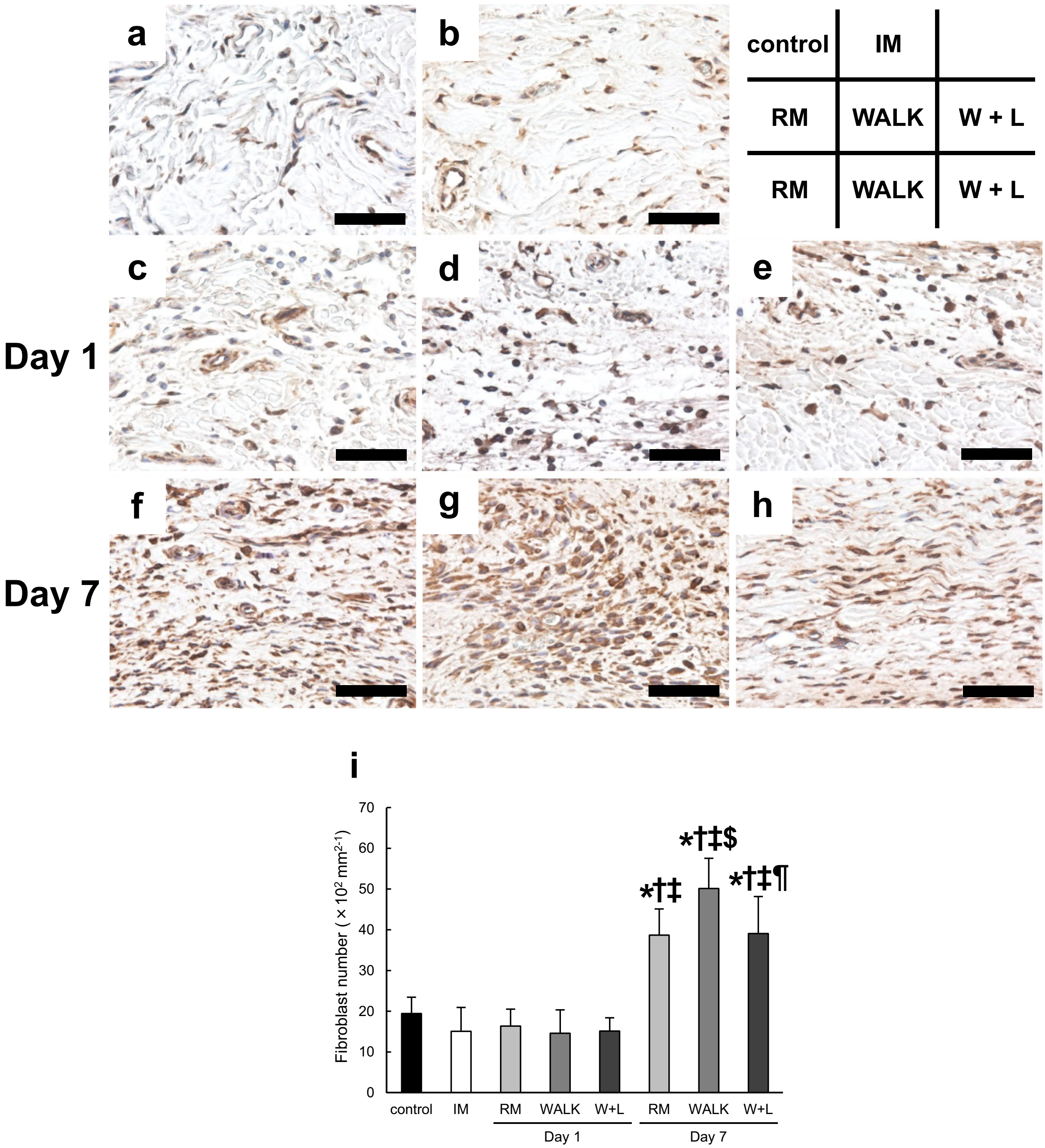

Fig. 6. Fibroblasts in the posterior joint capsule. Representative images of vimentin-stained postero-central knee joint capsules in the **a** control, **b** IM, **c** RM on day 1, **d** WALK on day 1, **e** W + L on day 1, **f** RM on day 7, **g** WALK on day 7, and **h** W + L on day 7 groups. **i** Fibroblast number. There was no difference in fibroblast number between the control (**a**) and IM groups (**b**, **i**). After 1 day of remobilization, the number of fibroblasts remained unchanged in all three groups (**c**–**e**, **i**). After 7 days of remobilization, the number of fibroblasts apparently increased in all three groups (**f**–**i**). Fibroblast number in the WALK group (**g**) was significantly higher compared with the RM group (**f**, **i**). In the W + L group (**h**), the number of fibroblasts was significantly lower than in the WALK group (**g**) and similar to the RM group (**f**, **i**). Scale bars = 50 μm. Values are mean ± standard deviation. *, significant difference compared with the control group ($P < 0.05$). †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$). ¶, significant difference compared with the WALK group at the same time point ($P < 0.05$).

6f–h). Among the three groups, fibroblast number in the WALK group (Fig. 6g) was significantly higher than in the RM (Fig. 6f) and W + L (Fig. 6h) groups ($P < 0.005$).

Gene Expression

Expression of the pro-inflammatory mediator *IL-1β* gene was not significantly different between the control and IM groups ($P = 0.18$; Fig. 7a). After 1 day of remobilization, gene expression of *IL-1β* in the RM group was significantly upregulated compared with the control group ($P < 0.001$). In the WALK group, *IL-1β* expression was further increased to three-fold of the RM group ($P < 0.001$). In the W + L group, gene expression of *IL-1β* was significantly lower than in the WALK group ($P < 0.001$) and did not differ from the RM group ($P = 1.00$); this value was still higher than in the control group ($P = 0.02$). After 7 days of remobilization, gene expression of *IL-1β* in all three groups decreased to match the IM group ($P > 0.80$).

Gene expression of pro-fibrotic cytokine *TGF-β1* in the IM group was not upregulated compared with the control group ($P = 1.00$; Fig. 7b). After 1 day of remobilization, *TGF-β1* gene expression in the WALK and W + L groups, but not in the RM group, was significantly increased compared with the control and IM groups ($P < 0.03$). Among the three groups, *TGF-β1* gene expression in the WALK and W + L groups was significantly higher than in the RM group ($P < 0.004$). After 7 days of remobilization, *TGF-β1* gene expression in the WALK group remained significantly higher than the control and IM groups ($P < 0.004$). In the W + L group, *TGF-β1* gene expression was significantly lower than in the WALK group ($P = 0.001$) and was not different from control and IM groups ($P > 0.92$).

Gene expression of *COL1A1* in the IM group did not differ from the control group ($P = 0.98$; Fig. 7c). After 1 day of remobilization, *COL1A1* gene expression levels in the RM and WALK groups remained unchanged compared with the control and IM groups ($P > 0.63$). In the W + L groups, *COL1A1* gene expression was significantly lower than the control and IM groups ($P < 0.01$). After 7 days of remobilization, *COL1A1* gene expression in the RM group did not significantly differ from the control and IM groups ($P > 0.47$). The WALK group, however, was significantly higher than the control and IM groups ($P < 0.002$). *COL1A1* gene expression in the WALK group was significantly higher than the RM group ($P = 0.01$). *COL1A1* gene expression in the W + L group was significantly lower than both the RM and WALK groups ($P < 0.03$) and did not differ from the control and IM groups ($P > 0.98$).

COL3A1 gene expression in the IM group also did not differ from the control group ($P = 0.07$; Fig. 7d). After 1 day of remobilization, *COL3A1* gene expression in the RM group was significantly lower than in the control group ($P = 0.045$). In the WALK group, *COL3A1* gene expression was not significantly different from the control and IM groups ($P > 0.16$). Conversely, *COL3A1* gene expression in the W + L group was significantly lower than the control and IM groups ($P < 0.02$). After 7 days of remobilization, *COL3A1* gene expression in the RM group did not differ from the control and IM groups ($P > 0.08$). In the WALK group, however, *COL3A1* gene expression was significantly upregulated compared with the control and IM groups ($P < 0.02$). In the W + L group, *COL3A1* gene upregulation was suppressed ($P = 0.02$) and was not different from the control and IM groups ($P > 0.55$).

DISCUSSION

In this study, we determined whether anti-inflammatory/anti-fibrotic treatment using LLLT combined with exercise can promote recovery from joint contracture without arthrogenic contracture progression. As a result, LLLT with treadmill walking did not alleviate total contracture initialized by 3 weeks immobilization followed by remobilization. However, LLLT prevented the progression of arthrogenic contracture as represented by prevention of the deterioration in the a-ROM during the remobilization period, which was characterized by inhibited fibrotic changes following inflammation in the joint capsule.

ROM was increased by myotomy in all groups. Here, we consider that knee flexors initially limit knee extension, and total contracture mainly reflects muscular-origin contracture. Immobilization-induced total contracture was partially and equally reduced by 7 days of remobilization in all three groups. In contrast, immobilization-induced arthrogenic contracture was further enhanced by 7 days of remobilization, and was promoted by treadmill walking. However, LLLT prevented the development of arthrogenic contracture caused by spontaneous ambulation and treadmill walking intervention during remobilization.

In this study, m-ROM was always smaller than a-ROM under all conditions, indicating that a-ROM restriction was not a determining factor of overall ROM in our study. The role of a-ROM restriction in limiting joint movement may be more evident during pronounced parts

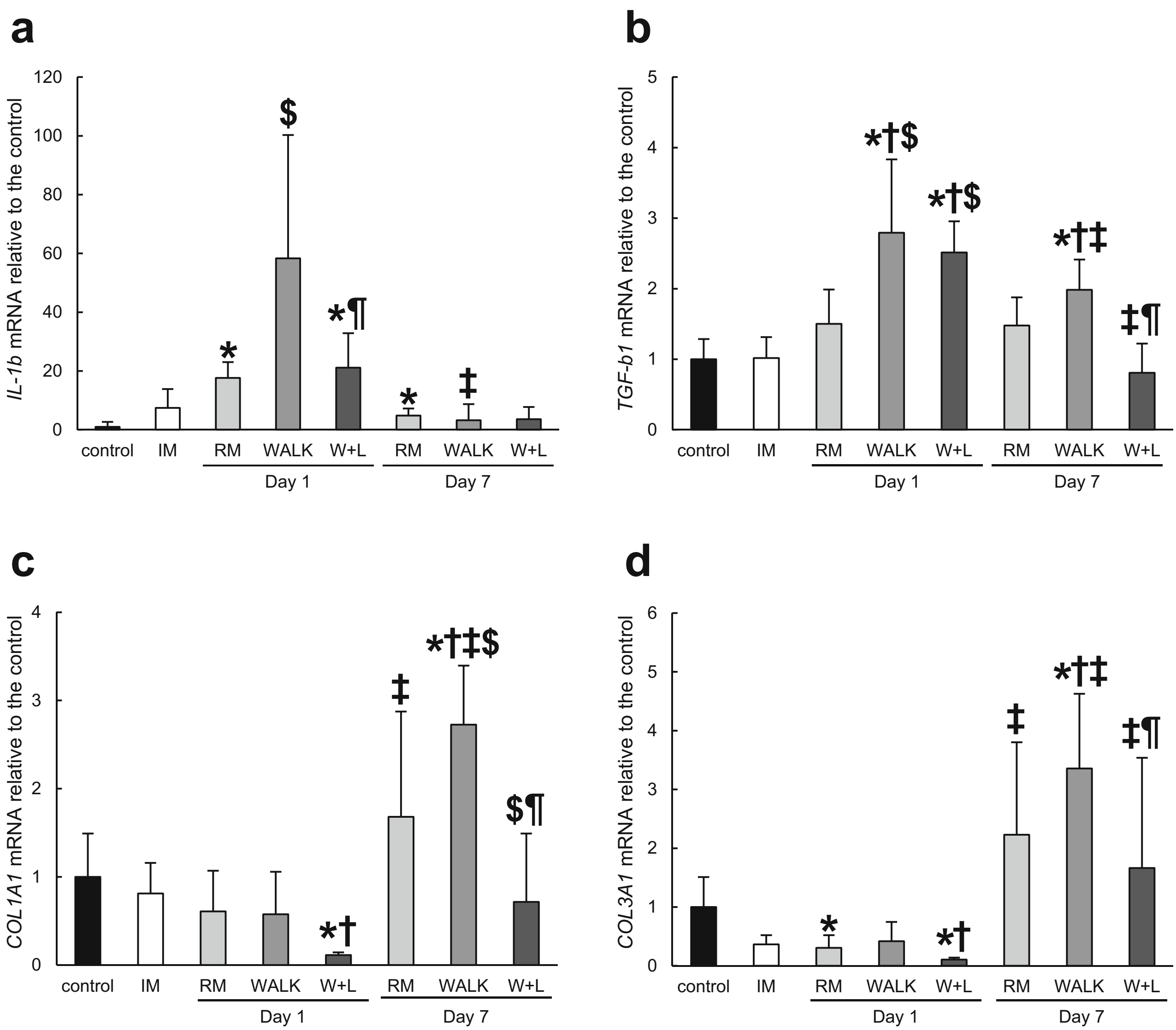

Fig. 7. Gene expression levels of inflammatory or fibrosis-related mediators. **a** After 1 day of remobilization, *IL-1β* gene expression in the RM group was significantly higher than in the control group. In the WALK group, expression of *IL-1β* further increased. In the W + L group, expression of *IL-1β* attenuated and maintained similar to the RM group level, although expression was still significantly higher than the control group. **b** *TGF-β1* gene expression in WALK and W + L groups was significantly upregulated after 1 day of remobilization. After 7 days of remobilization, significant upregulation of the *TGF-β1* gene persisted in the WALK group. However, *TGF-β1* gene expression in the W + L group was significantly lower than in the WALK group and not different from the control and IM groups. **c** *COL1A1* gene expression was significantly downregulated in the W + L group after 1 day of remobilization. After 7 days of remobilization, *COL1A1* gene expression was significantly upregulated in the WALK group, but this upregulation was prevented in the W + L group. **d** *COL3A1* gene expression was also significantly downregulated in the W + L group after 1 day of remobilization. After 7 days of remobilization, *COL3A1* gene expression in the WALK group was significantly upregulated, which was attenuated in the W + L group. Values are mean ± standard deviation. *, significant difference compared with the control group ($P < 0.05$). †, significant difference compared with the IM group ($P < 0.05$). ‡, significant difference compared with the same group after 1 day of remobilization ($P < 0.05$). $, significant difference compared with the RM group at the same time point ($P < 0.05$). ¶, significant difference compared with the WALK group at the same time point ($P < 0.05$).

of the m-ROM recovery period. Trudel et al. [12] reported that remobilization resolved immobilization-induced myogenic contracture in a rat model, but arthrogenic contracture did not recover spontaneously, which determined the final recovery period. Thus, preventing arthrogenic contracture progression by LLLT will likely lead to a better final recovery of m-ROM. To confirm this, more long-term follow-up studies are needed in the future.

In a previous study, we revealed that LLLT without exercise during remobilization also prevented the progression of remobilization-induced arthrogenic contracture [16]. However, exercise during remobilization may be effective for improving daily activities by increasing muscle strength [23–25]. Therefore, the combination of LLLT and exercise may meet the requirement for proper recovery of muscle strength and daily activities by exercise without progression of arthrogenic contracture, and it may be clinically viable and an option for rehabilitating remobilized joints.

We previously showed that 7 days of remobilization following 3 weeks immobilization progressed arthrogenic contracture, and that treadmill walking during remobilization further enhanced arthrogenic contracture [13]. In this study, we reproduced arthrogenic contracture progression in the RM group after 7 days of remobilization. The arthrogenic contracture progression was accompanied by fibrosis in the posterior joint capsule with increased collagen density due to accumulation of both type I and III collagens. We also often observed obstructed synovial folds occupied by fibrous tissues, which induced synovium shortening. Treadmill walking during remobilization enhanced fibrotic responses, which were characterized by increased pro-fibrotic genes, as well as proteins, such as the cytokine *TGF-β1* gene and type I and III collagen genes and proteins, resulting in increased collagen density and joint capsule thickness. Tissue extensibility is generally determined by length and stiffness. Shortening of the synovium, which lines the joint capsule, contributes to remobilization-induced arthrogenic contracture progression. However, synovial length is unlikely to contribute to further arthrogenic contracture progression from exercise associated with treadmill walking, as there was no difference in synovial length between the RM and WALK groups. Connective tissue stiffness, which may affect the extensibility of the joint capsule, is determined by various interacting factors, including collagen content [26] and cross-sectional area [27]. Thus, increased collagen density at 7 days of remobilization contributes to remobilization-induced arthrogenic contracture progression. Treadmill walking further induced joint capsule thickening and increased collagen density, which further explains the progression of arthrogenic contracture in the WALK group compared with the RM group.

Arthrogenic contracture progression induced by remobilization and treadmill walking was completely prevented by LLLT during remobilization (a-ROM in the W + L group was maintained at a similar level to the IM group). After 7 days of remobilization, expression of pro-fibrotic *TGF-β1*, *COL1A1*, and *COL3A1* genes in the W + L group was significantly lower than in the WALK group. Consequently, expressions of both type I and III collagen proteins and collagen density were lower in the W + L than in the WALK group and were comparable to those of the RM group. Therefore, it is reasonable to assume that LLLT prevented arthrogenic contracture progression by suppressing the inflammation-fibrosis cascade. However, although fibrosis-related outcomes, such as joint capsule thickness, collagen density, and type I and III collagen protein expression, were similar between the RM and W + L groups, arthrogenic contracture was significantly milder in the W + L than in the RM group. Thus, attenuation of fibrotic reactions in the posterior joint capsule cannot solely explain the prevention of arthrogenic contracture progression by LLLT. In this study, we focused our analyses on the posterior joint capsule following a previous study that indicated that the posterior joint capsule primarily contributes to the formation of immobilization-induced knee flexion contracture [28]. However, other joint components, such as ligaments, are also affected by joint immobilization and remobilization and potentially contribute to the degree of arthrogenic contracture [28–30]. In addition, qualitative alterations, such as cross-links between collagen fibers and collagen fiber bundle orientation, can also contribute to changes in tissue stiffness [26, 31]. More broad and detailed analyses will further clarify the mechanisms underlying the beneficial effects of LLLT on arthrogenic contracture.

Previous studies suggest that arthrofibrosis is triggered by intra-articular inflammation [9, 13, 32]. In addition, we recently demonstrated that administration of the steroidal anti-inflammatory drug dexamethasone during the remobilization period prevents remobilization-induced joint capsule fibrosis [14]. In this study, we observed inflammatory reactions in the joint capsule characterized by upregulation of the pro-inflammatory cytokine *IL-1β* gene, as well as inflammatory cell infiltration after 1 day of remobilization in the RM group, as per previous studies [9, 13]. Moreover, treadmill walking further increased *IL-1β* gene expression, indicating enhanced inflammatory reactions. Following LLLT intervention during treadmill walking, *IL-1β* gene expression was attenuated and maintained at a similar level to the RM group. Previous studies have also reported the anti-inflammatory effects of LLLT on joint structure. Alves et al. [33] reported that LLLT for arthritic joints decreased gene expression of pro-inflammatory cytokines, including *IL-1β*. LLLT also inhibited gene and protein expression of IL-1β in synoviocytes obtained from rheumatoid arthritis patients [34].

IL-1 plays a key role in the development of arthrofibrosis. Intra-articular injection of an IL-1 receptor antagonist actually improves ROM in patients with knee arthrofibrosis [35]. Moreover, administration of an IL-1 receptor antagonist inhibits synovial fibrosis secondary to antigen-induced arthritis [36]. Recently, Dixon et al. [37] suggested that IL-1 induces arthrofibrosis development *via* fibroblasts, which express high levels of the IL-1 receptor. Fibroblasts proliferate during arthrofibrosis formation and produce extracellular matrix proteins including collagens [13, 38]. Since IL-1β stimulates proliferation of synovial fibroblasts in arthritic joints [39], upregulation of IL-1β may contribute to the formation of joint capsule fibrosis by proliferating fibroblasts. In fact, remobilization-induced hypercellularity was mainly composed of increased fibroblasts following *IL-1β* upregulation. Fibroblast proliferation and *IL-1β* upregulation in the joint capsule were further facilitated by treadmill walking. Conversely, LLLT with treadmill walking partially attenuated hypercellularity as well as *IL-1β* upregulation. Accordingly, a previous study also reported that LLLT for rat arthritic joints reduced both pro-inflammatory cytokine and fibroblast-like synoviocytes [40]. Thus, anti-fibrotic effects by LLLT could be exerted, at least partially, through anti-inflammatory effects.

Seven days of treadmill walking with and without LLLT did not promote recovery of total contracture, as represented by m-ROM restriction. It is controversial whether treadmill exercise during remobilization is effective for recovery from joint contractures. One study reported that 1 week of treadmill exercise did not promote recovery of m-ROM [13], while others demonstrated that 2 or 6 weeks of treadmill exercise showed improved m-ROM recovery [6, 7]. Therefore, the remobilization period in the present study may not be long enough to allow sufficient recovery of total contracture. More long-term intervention may be needed to promote m-ROM recovery by treadmill exercise. In addition, the duration of immobilization may also influence the effect of treadmill exercise on joint contracture recovery. Previous studies indicated improved m-ROM levels from treadmill exercise during remobilization; here, duration of joint immobilization was shorter than our study (2 *vs.* 3 weeks) [6, 7]. Prolonged immobilization may inhibit the benefits of treadmill exercise on joint contracture.

This study has limitations. Identification of cell types was important to demonstrate inflammatory and fibrotic conditions. In this study, inflammatory cells were identified by only circular morphology. Identification of inflammatory cells by immunohistochemical markers would provide more convincing conclusions. We identified fibroblasts based on vimentin expression and spindle-shaped morphology. To clearly distinguish fibroblasts from other types of cells, combination use of multiple markers such as CD45 (a marker for leukocyte), CD31 (a marker for endothelial cell), and mEF-SK4 (a marker for fibroblast) [41] would be needed.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. No conflicts of interest, financial or otherwise, are declared by the authors.

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