Supplementary File

Method 1

Hindlimb suspension

Rats in the HS and HS-OA groups were subjected to tail suspension for 4 weeks. Hindlimb suspension was performed according to the tail-suspension method described in our previous studies[1–3]. Briefly, under inhalation anesthesia with isoflurane, the tail of each rat was disinfected. A sterile steel wire was then used to drill into the proximal coccyx in which the wire remained and was shaped into a ring. The tail ring was then connected to a track hung above the cage by using another wire, thereby enabling the animals to move freely on their forelimbs in the cage. The head-down tilt angle was monitored throughout the experimental period and remained at approximately 30° [4].

Surgical induction of OA

The same highly experienced operators (IT and KT) performed all DMM surgeries. The DMM was induced by transecting the medial meniscotibial ligament (MMTL) in the left knee joint as described previously[5–7]. Anesthesia was induced via isoflurane inhalation, and both knees were shaved and disinfected. Both knee joints were exposed

after making a 3-cm skin incision and a 1-cm medial capsular incision with a #15 blade (Feather Safety Razor Co., Ltd., Osaka, Japan). When it was difficult to secure the visual field because of bleeding, the bleeding was stopped by pressing the bleeding site with a swab soaked with adrenaline (Bosmin solution; Daiichi Sankyo Co., Ltd., Tokyo, Japan)[5]. While the patellar tendon and fat pad were pulled and shifted laterally with forceps (without dislocating the patella), we confirmed that the MMTL was exposed. Then, the MMTL was transected with a #15 blade, and with each surgical procedure, we confirmed that the medial meniscus had displaced medially and that the instability had been induced. After thoroughly washing the surgical site with saline, we closed the articular capsule and skin incisions with 5-0 nylon (Akiyama Medical Manufacturing Co., Ltd., Tokyo, Japan). For internal controls, a sham operation was performed on the right knee joint by using the same approach without MMTL transection. A previous study reported that there was no significant difference in OA severity between sham operated and age-matched unoperated control joints[8].

Histomorphometrical analyses

To evaluate the cartilage thickness, intensity of matrix staining with toluidine blue, chondrocyte density, and osteophyte length, we used Adobe Photoshop CC imaging

37 software (Adobe Systems, Inc., San Jose, CA, USA) and measured these parameters[2,4,9].
38 For evaluation of cartilage thickness, staining intensity and chondrocyte density in the
39 OA and HS-OA groups, 1 section with the highest OARSI score among 3 sections was
40 chosen. For evaluation of osteophyte length in the OA and HS-OA groups, 1 section
41 with the longest osteophyte among 3 sections was chosen.
42 To evaluate cartilage thickness, digitized images of the sections stained with toluidine
43 blue were used[1–3]. The cartilage thickness was defined as the distance between the
44 cartilage surface and the osteochondral junction. We measured the area of the cartilage
45 with a width of 1 mm in the center of the lesion at the tibia in the medial tibiofemoral
46 joint and used this value as the average cartilage thickness. In the CON and HS groups,
47 we measured the area of the cartilage with a width of 1 mm in the center at the tibia in
48 the medial tibiofemoral joint.
49 To evaluate matrix intensity, digitized images of cartilage stained with toluidine blue
50 were converted to greyscale (white, 255; black, 0) to assess the relative intensity of
51 toluidine blue staining. The average staining intensity was calculated at the same area in
52 the same manner as performed for the measurement of articular cartilage thickness.
53 To evaluate chondrocyte density, digitized images of the sections stained hematoxylin
54 and eosin were used. Chondrocyte density was determined as the number of

55 chondrocytes per unit area of cartilage. This unit area was calculated by using the same
56 method as used for the above cartilage thickness, and the width to be measured was set
57 to 200 μm. Chondrocytes with visible nuclei within the area of interest were counted
58 manually.

59 To evaluate osteophyte formation of the tibia in the medial tibiofemoral joint, we
60 used digitized images of sections stained with toluidine blue and measured the length
61 from the deepest point of its base at the chondro-osseous junction to the surface of the
62 overlying cartilage at its thickest point[10].

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64 References

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Method 2

Grade	Description
0	No changes.
1	Increased basophilia at tidemark. No fragmentation of tidemark. No marrow changes or, if present, minimal and focal. Increased thickening of subchondral bone subjacent to the area of greatest articular cartilage lesion severity.
2	Increased basophilia at tidemark. Minimal to mild focal fragmentation of calcified cartilage of tidemark. Mesenchymal change in marrow (fibroblastic cells) involving approximately 1/4 of the subchondral region under the lesion. Increased thickening of subchondral bone subjacent to the area of greatest articular cartilage lesion severity.
3	Increased basophilia at tidemark. Mild to marked fragmentation (multiple larger areas) of calcified cartilage/subchondral bone loss Mesenchymal change in marrow in up to 3/4 of the total area. Areas of marrow chondrogenesis may be evident but no major collapse of articular cartilage into epiphyseal bone (definite depression in the surface).
4	Increased basophilia at tidemark. Marked to severe fragmentation of calcified cartilage.

	Marrow mesenchymal change involves up to 3/4 of the area. Articular cartilage has collapsed into the epiphysis to a depth of ≤250 mm from the tidemark (see definite depression in the surface cartilage).
	Increased basophilia at the tidemark. Marked to severe fragmentation of calcified cartilage.
5	Marrow mesenchymal change involves up to 3/4 of the area. Articular cartilage has collapsed into the epiphysis to a depth of >250 mm from the tidemark.

Calcified cartilage and subchondral bone damage score

Result 1. Changes in the body weights (g) of the rats throughout the experiment

Preliminary study of cartilage atrophy under the unloading condition.

	CON group	HS group
At experiment start	284.6 ± 5.8 (277.3–291.8)	287.2 ± 10.4 (274.1–300.2)
At 4 weeks	410.0 ± 15.0* (391.2–428.7)	344.6 ± 19.5*† (320.2–368.9)

At 2 weeks

	OA group	HS-OA group
At DMM	421.0 ± 14.3 (403.1–438.8)	359.8 ± 13.6‡ (342.8–376.7)
At 2 weeks	431.6 ± 17.3 (410.1–453.0)	376.8 ± 12.5‡ (361.8–392.4)

At 4 weeks

	OA group	HS-OA group
At DMM	378.4 ± 10.9 (364.8–391.9)	330.4 ± 14.3‡ (312.5–348.2)
At 4 weeks	429.0 ± 21.2 (402.5–455.4)	403.4 ± 16.4 (383.0–423.7)

* Significant differences from baseline for the same group.

† Significant differences from CON group at the same experimental period.

‡ Significant differences from OA group at the same experimental period.

$P < .05$ for all.

Result 2. Cartilage atrophy

Thickness of the articular cartilage (μm)

	CON group	HS group
At 4 weeks	395.1 ± 21.0 (380.1–410.1)	355.0 ± 19.7* (340.9–369.2)

Matrix staining by toluidine blue (pixel value)

	CON group	HS group
At 4 weeks	42.8 ± 11.0 (34.9–50.7)	61.2 ± 8.5* (55.1–67.3)

Mean ± SD (95% CI)

* Significant differences from CON group at the same experimental period.

$P < .05$ for all.

Result 3. Histological scores

Maximum OARSI score

	OA group	HS-OA group
At 2 weeks	4 (3–7.5)	6 (6–10.5)
At 4 weeks	8 (6–9)	16 (14–16) *
At 8 weeks	12 (10.5–12)	16 (12–16) *

Summed OARSI score

	OA group	HS-OA group
At 2 weeks	8 (5.5–11)	12 (10.5–15) *
At 4 weeks	17 (15.5–19)	22 (20–25) *
At 8 weeks	20 (17.5–26)	36 (29–40) *

Subchondral bone damage score

	OA group	HS-OA group
At 2 weeks	0 (0–0)	0 (0–0)
At 4 weeks	0 (0–1)	0 (0–1)
At 8 weeks	1(0–1)	2(1–2.5) *

Median (lower quartile–upper quartile)

* Significant differences from Con group during the same experimental period.

$P < .05$ for all.

Supplementary Result 4. Histomorphometrical analyses

Cartilage thickness (μm)

	OA group		HS-OA group	
	Operated	Sham	Operated	Sham
At 2 weeks	332.7 ± 27.2 (298.9–366.5)	428.0 ± 30.0** (390.7–465.4)	264.6 ± 18.7* (241.3–287.9)	424.8 ± 32.4** (384.6–465.1)
At 4 weeks	253.2 ± 39.7 (203.9–302.6)	391.1 ± 33.3** (349.7–432.5)	249.9 ± 48.3 (189.9–309.9)	397.7 ± 10.4** (384.7–410.7)
At 8 weeks	261.1 ± 46.3 (203.5–318.6)	360.7 ± 34.4** (317.9–403.5)	233.6 ± 27.9 (198.8–268.3)	385.2 ± 17.0** (364.0–406.3)

Matrix intensity (pixel value)

	OA group		HS-OA group	
	Operated	Sham	Operated	Sham
At 2 weeks	44.5 ± 17.6 (22.6–66.4)	40.3 ± 9.0 (29.1–51.5)	65.3 ± 9.2* (53.9–76.8)	42.8 ± 2.0** (40.3–45.4)
At 4 weeks	71.1 ± 25.6 (39.3–103.0)	41.1 ± 10.5** (28.0–54.3)	83.7 ± 13.5 (66.9–100.5)	35.9 ± 7.5** (26.5–45.4)
At 8 weeks	70.4 ± 13.3 (53.9–87.0)	37.0 ± 5.5** (30.1–43.9)	78.9 ± 24.4 (48.5–109.3)	34.0 ± 4.4** (28.4–39.6)

Chondrocyte density (cells/mm^2)

	OA group		HS-OA group	
	Operated	Sham	Operated	Sham
At 2 weeks	869.2 ± 92.4 (754.4–984.1)	1150.6 ± 186.1** (919.5–1381.7)	616.7 ± 59.3* (542.9–690.4)	1062.5 ± 57.1** (991.5–1133.4)
At 4 weeks	679.5 ± 160.8 (479.8–879.2)	1016.6 ± 98.8** (893.9–1139.4)	741.3 ± 169.0 (531.4–951.2)	1131.9 ± 88.5** (1022.0–1241.8)
At 8 weeks	670.9 ± 88.2 (561.2–780.5)	1120.6 ± 146.6** (938.5–1302.8)	555.8 ± 84.4 (451.0–660.7)	1155.1 ± 99.4** (1031.7–1278.6)

Osteophyte length (μm)

	OA group		HS-OA group	
	Operated	Sham	Operated	Sham
At 2 weeks	317.3 ± 76.8 (221.8–412.7)	181.5 ± 13.9** (164.1–198.8)	252.1 ± 52.6 (186.7–317.5)	164.7 ± 26.4 (131.8–197.5)
At 4 weeks	350.6 ± 55.4 (281.8–419.4)	201.8 ± 20.9** (175.8–227.9)	325.0 ± 31.9 (285.4–364.6)	215.1 ± 14.4** (197.2–233.0)
At 8 weeks	456.5 ± 73.1 (365.6–547.4)	176.4 ± 25.7** (144.4–208.4)	412.7 ± 107.2 (279.6–545.9)	155.5 ± 18.4** (132.6–178.4)

Mean ± SD (95% CI)

* Significant differences from OA group during the same experimental period.

** Significant differences from operated limb of the same group during the same

181 experimental period.

182 $P < .05$ for all.