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(Citation)
International Orthopaedics,41(6):1211-1217

(Issue Date)
2017-06

(Resource Type)
journal article

(Version)
Accepted Manuscript

Altered Expression of SDF-1 and CXCR4 during Fracture Healing in Diabetes Mellitus

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Abstract

Purpose Diabetes mellitus (DM) is known to impair fracture healing. The purpose of this study was to elucidate and compare the gene expression patterns and localization of stromal cell-derived factor 1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) during fracture healing of the femur in rats with and without DM.

Methods Closed transverse fractures were created in the femurs of rats equally divided into a DM group and control group; DM was induced by streptozotocin. At post-fracture days 5, 7, 11, 14, 21, and 28, total RNA was extracted from the fracture callus and mRNA expression levels of SDF-1 and CXCR4 were measured by real-time polymerase chain reaction. Localization of SDF-1 and CXCR4 proteins at the fracture site was determined by immunohistochemistry at days 21 and 28.

Results SDF-1 expression was significantly lower in the DM group than in the healthy group on days 21 and 28, and showed a significant difference between days 14 and 21 in the healthy group. There was no significant difference in CXCR4 expression levels between the healthy and DM groups at any time point. On day 21 immunoreactivity of SDF-1 and CXCR4 was detected at the fracture site of the healthy group but no immunoreactivity was observed in the DM group. On day 28, immunoreactivity of SDF-1 and CXCR4 was detected at the fracture site in both groups.

Conclusion Gene expression and localization of SDF-1 and CXCR4 was altered during fracture healing, which may contribute to the impaired fracture healing in DM.

Keywords: Diabetes mellitus; Fracture healing; Delayed union; Endochondral ossification; Stromal cell-derived factor 1; CXC chemokine receptor 4

Introduction

Diabetes mellitus (DM) is a worldwide problem and one of the most prevalent chronic diseases. By the year 2040, 642 million people are estimated to be suffering from the disease [1]. Diabetes adversely affects bone health and is associated with reduction of bone strength, resulting in a higher risk of fracture [2]. Clinical studies have shown a significantly higher incidence of delayed union, nonunion, and a doubling of the time to fracture healing in diabetic compared with non-diabetic patients [2–4]. Indeed, DM patients showed prolongation of the fracture healing time by up to 87% [3]. Although the association between DM and impaired fracture healing, including delayed union and nonunion, has been documented, few studies have explored the detailed molecular mechanisms by which DM affects the process of fracture healing [5].

Stromal cell-derived factor 1 (SDF-1) is a member of the pro-inflammatory CXC chemokine family and plays a role in cell survival, growth, and development through the activation of a G-protein-coupled transmembrane receptor, CXC chemokine receptor 4 (CXCR4) [6, 7]. SDF-1 is upregulated in injured tissue, and the SDF-1/CXCR4 axis plays important roles in progenitor homing, hematopoiesis, angiogenesis, and wound healing. SDF-1 is involved in the chemotactic recruitment of a number of cell types, including mesenchymal progenitor cells and hematopoietic stem cells. Increasing evidence suggests that the SDF-1/CXCR4 axis plays a crucial role in fracture healing by regulating the recruitment and differentiation of stem and progenitor cells at fracture sites [8]. Some studies have implicated low levels of SDF-1 expression in diabetic wound tissues as one of the potential mechanisms involved in the impaired wound healing in DM [9]. However, the role of the SDF-1/CXCR4 axis and the expression of SDF-1 and CXCR4 during fracture healing in DM remains unclear. Therefore, the purpose of this study was to elucidate and compare

the temporal gene expression patterns and localization of SDF-1 and CXCR4 during fracture healing of the femur of rats with and without DM.

Materials and Methods

Animals

A total of 100 10-week-old male Sprague–Dawley rats (SLC, Hamamatsu, Japan) were used in this study, which were randomly assigned to the DM group (n = 50) and healthy control group (n = 50). As an impaired fracture healing model, a DM rat was established by a single intravenous injection of 40 mg/kg streptozotocin (STZ: Sigma, St. Louis, MO, USA) [10]. This experimental model reproducibly leads to type 1 DM. Rats with blood glucose levels over 300 mg/dl at 1 week after injection were used for experiments, and fractures were made 2 weeks after STZ injection. Healthy control rats were injected with sodium chloride as a sham treatment.

Surgical procedure

Closed transverse femoral shaft fractures were created in both groups. The details of these procedures have been previously described [11]. In brief, a 1.25-mm-diameter Kirschner wire was inserted retrogradely into the right femoral intramedullary canal, and a closed transverse femoral shaft fracture was produced using a three-point bending apparatus with a drop weight.

Radiographic assessment of fracture repair

At post-fracture days 7, 14, 21, and 28, eight animals in each group were anesthetized and fixed in the supine position with the limbs fully extended, and radiographs of the fracture site were acquired. On radiographic evaluation, each callus on four cortices (two on the antero-posterior and two on the lateral radiograph) were evaluated by two orthopedic surgeons blinded to the group, and a bony union was defined when three of four cortices were bridged and/or fracture lines disappeared completely [12].

Histology of fracture sites and assessment of cartilage area

At days 7, 14, 21, and 28 after fracture, the fractured femur was harvested from five animals in each group. The femur was fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin wax. Sagittal sections were cut and stained with safranin-O/fast green for histological examination and assessment of the cartilage area. Measurements of the cartilage area at the fracture sites were performed with the image analysis software Image J [13].

Assessment of angiogenesis

At days 7, 14, 21, and 28 after fracture, the cross-sectional capillary density at the perifracture site was evaluated in five animals in each group. The sections were immunohistochemically stained with fluorescein-labeled isolectin B4 (Vector Laboratories, Burlingame, CA, USA), an endothelial cell marker. Nuclear staining was performed with 4′ ,6-diamidino-2-phenylindole solution

(Nacalai Tesque, Kyoto, Japan). Capillaries were morphometrically examined under a fluorescent microscope, counted in five randomly selected fields in the callus tissue, and averaged.

Quantitative real-time polymerase chain reaction (PCR) analysis

Five animals from each group were euthanized at post-fracture days 5, 7, 11, 14, 21, and 28 for real-time PCR. At each time point, the external callus generated at the fracture site was excised circumferentially from the underlying intact cortical bone by dissection with a scalpel and rongeur. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and was reverse-transcribed into single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using StepOne Sequence Detector and SYBR Green reagents (both from Applied Biosystems) following the recommended protocols. All primer sequences are shown in Table 1. The relative abundance of each mRNA was calculated using the comparative ΔΔCT method, and is presented as a fold change relative to levels in the post-fracture day 7 healthy sample [14].

Immunohistochemistry for SDF-1 and CXCR-4

The sections of the femora harvested on post-fracture days 21 and 28 (five animals per group) were incubated overnight at 4 °C with the primary antibodies (rabbit polyclonal antibody against SDF-1 and CXCR4, respectively [1:100 dilutions; both from Santa Cruz Biotechnology, TX, USA]), and subsequently treated with peroxidase-labeled anti-rabbit immunoglobulin (Histofine Simplestain max PO (R), Nichirei Bioscience, Tokyo, Japan) at room temperature for 60 min. The

signal was developed as a brown reaction product using the peroxidase substrate 3-amino-9-ethylcarbazole (Histofine Simplestain AEC Solution, Nichirei Bioscience). The sections were counterstained with hematoxylin.

Statistical analysis

Quantitative data are presented as the mean and standard deviation. The chi-square test was used to compare the radiographic results between the groups at each time point. The values of the DM and healthy groups were compared at each time point using the Mann–Whitney U-test. The Kruskal-Wallis test and Mann-Whitney U-test with Bonferroni correction were used to compare data between time points in both groups. A p-value of <0.05 was defined as statistically significant.

Results

Radiographic assessment of fracture repair

At day 14, anchoring calluses were observed in the healthy group but not in the DM group (Fig. 1). At day 21, enlargement of the callus was observed and three (37.5%) of the animals had achieved fracture union in the healthy group. In contrast, despite anchoring calluses, no animals achieved union in the DM group. At day 28, seven (87.5%) of the animals in the healthy group had achieved union compared with no animals in the DM group. The union rates of the two groups on days 21 and 28 were significantly different ($p < 0.05$).

Histology of fracture sites

Fig. 2a shows the histology of fracture healing in healthy rats and DM rats on days 7, 14, 21, and 28. On day 14, animals in the healthy group had formed a thick callus consisting of chondrocytes and newly formed woven bone, whereas smaller cartilage was developed in the DM group. In the healthy group on day 21, the two calluses on each side of the fracture were nearly united, and newly formed woven bone predominated in the callus. In contrast, rats in the DM group exhibited poor bridging callus formation at the fracture site. Finally, on day 28, the callus in the healthy group had nearly united and active remodeling was underway. In contrast, cartilage remained between the woven bones in the DM group, suggesting delayed union.

The cartilage area was significantly lower in the DM group than that in the healthy group on days 14 and 21 ($p < 0.05$; Fig. 2b). In the healthy group, the cartilage area increased over time, with a peak on day 14 followed by a decline. The cartilage area of the DM group remained at a lower level over the course of healing. There was no statistically significant difference in cartilage area across time in either group.

Capillary density at perifracture sites

Vascular staining with isolectin B4 showed that the capillary density in the callus tissue on day 28 was significantly lower in the DM group than in the heathy group ($p < 0.05$; Fig. 3).

Gene expression levels of SDF-1 and CXCR4

The mRNA expression level of SDF-1 was significantly lower in the DM group than that in the healthy group on days 21 and 28 ($p < 0.05$; Fig. 4a). In the healthy group, SDF-1 expression increased with time and peaked on day 28; there was a significant difference between days 14 and 21 ($p < 0.05$). In contrast, the DM group showed relatively constant SDF-1 expression over the course of healing with no significant difference in expression levels among each time point.

There was no significant difference in CXCR4 expression levels between the healthy and DM groups at any time point (Fig. 4b). CXCR4 expression in both groups showed a relatively constant level over the course of healing, with no statistically significant difference among each time point in either group.

Immunolocalization of SDF-1 and CXCR4 at fracture sites

On day 21 in the healthy group, immunoreactivity of SDF-1 and CXCR4 was detected in proliferative chondrocytes and periosteal cells, and in osteoblasts lined on the trabecular bone (Fig. 5a). In contrast, no immunoreactivity of SDF-1 and CXCR4 was observed in the DM group. On day 28, immunoreactivity of SDF-1 and CXCR4 was detected in periosteal cells, and in the osteoblasts lined on the trabecular bone in both groups (Fig. 5b). Proliferating chondrocytes were still present in the DM group on day 28. In contrast, in the healthy group, proliferating chondrocytes were not present, and showed immunoreactivity of SDF-1 and CXCR4. The immunolocalization patterns of SDF-1 and CXCR4 are summarized in Table 2.

Discussion

DM has been shown to impair fracture healing in both clinical and experimental settings [2, 15]. Several studies have indicated that the STZ-induced diabetic rat serves as a useful model for studying fracture healing in DM [10, 16]. Long-bone fractures of STZ-induced diabetic animals exhibit smaller calluses with decreased bone and cartilage formation, proliferation and differentiation of osteoblasts and chondrocytes, and mechanical strength [10, 13, 16, 17]. In agreement with these previous studies [10, 13, 16], in the current study, the rate of fracture-healing in diabetic rats decreased and there was a significant difference in the union rate at days 21 and 28 between the healthy and DM groups. Histological analysis also demonstrated a prolonged fracture healing process in the DM group that was characterized by a smaller cartilage callus and a delay in endochondral ossification.

The SDF-1/CXCR4 axis plays a pivotal role in fracture healing by affecting the migration and differentiation of progenitor cells at fracture sites [8, 18, 19]. Kitaori et al. [19] reported that SDF-1 is induced in the fractured bone and promotes endochondral bone formation by recruiting mesenchymal progenitor cells to the site of injury. In the present study, we found altered temporal gene expression patterns and localization of SDF-1 and CXCR4 at the fracture sites between the healthy and DM groups. Real-time PCR analysis revealed that the expression level of SDF-1 increased over time in the healthy group, which did not occur in the DM group. By contrast, significant downregulation in SDF-1 was observed in the DM group on days 21 and 28 compared with the healthy group. In the healthy group on day 21, both SDF-1 and CXCR4 immunoreactivity was detected in proliferating chondrocytes, osteoblasts, and periosteal cells, whereas neither SDF-1 nor CXCR4 immunoreactivity was detected within the fracture callus in the DM group at this point. On day 28, both SDF-1 and CXCR4 immunoreactivity was detected in these cells in both groups. This is the first study to demonstrate a change in the local expression of SDF-1 and CXCR4

during fracture healing in DM.

DM impairs endochondral ossification, an essential component of fracture healing, by diminishing chondrogenic cellular proliferation and cartilage-related collagen synthesis, and delayed terminal differentiation of chondrocytes, resulting in a smaller callus size [10, 16]. In the present study, the cartilage area within the callus in the DM group at days 14 and 21 was significantly smaller than that in the healthy group, which is consistent with previous reports [10, 13]. The SDF-1/CXCR4 axis has been reported to regulate chondrocyte differentiation during endochondral bone ossification [20, 21]. Murata et al. [21] reported that SDF-1 is crucial to the hypertrophic conversion and subsequent calcification of chondrocytes. Thus, the absence of up-regulation of SDF-1 expression from day 14 through 28 in rats with DM might contribute to the disturbed progression of endochondral ossification. It is possible that this alteration may impede the fracture healing process in the DM group.

Delay or compromise to the angiogenic process during fracture healing has a significant effect on the progression of proper bone healing. Complications of DM are characterized by vasculopathy associated with aberrant angiogenesis [2, 22], and inhibited angiogenesis/vasculogenesis contributes to the poor healing of diabetic wounds. Although an association of diminished angiogenesis/vasculogenesis with impaired fracture healing in DM has been suggested, there has been no study investigating vascularity at fracture sites during fracture healing in DM. For the first time, we found that the capillary density at perifracture sites on day 28 was significantly lower in the DM group than in the healthy group. Moreover, the SDF-1/CXCR4 axis acts as a key regulator of angiogenesis and contributes to the regulation of endothelial progenitor cell (EPC) recruitment in ischemic tissues [23]. Kawakami et al. [24] demonstrated that the role of the SDF-1/CXCR4 axis in the mobilization and incorporation of EPCs is an important mechanism for fracture healing.

EPCs have been shown to be involved in physiological and pathological angiogenesis/vasculogenesis [25]. The reduced expression of SDF-1 on days 21 and 28 in the DM group may have contributed to disturbed angiogenesis/vasculogenesis at fracture sites through insufficient recruitment of EPCs, thus potentially leading to delayed fracture healing.

In conclusion, gene expression and localization of SDF-1 and CXCR4 at fracture sites were altered during fracture healing in experimental diabetes, which may contribute to the impaired fracture healing in association with inhibition of endochondral ossification and angiogenesis. Our study provides new information to help understand the underlying pathogenesis of impaired fracture healing in DM. Although further *in vivo* functional analyses are needed, including gain-of-function tests in the fracture healing of diabetic rats, our findings may lead to the development of a new strategy for the therapeutic use of SDF-1 to promote fracture healing or prevent nonunion in patients with DM.

Acknowledgments

The authors would like to give a special thanks to Mr. T. Ueha, Ms. M. Yasuda, Ms. K. Tanaka, and Ms. M. Nagata (Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine) for their excellent technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

Funding There is no funding source.

Ethical approval All animal procedures were performed under the approval and guidance of the Animal Care and Use Committee of Kobe University Graduate School of Medicine.

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Figure Legends

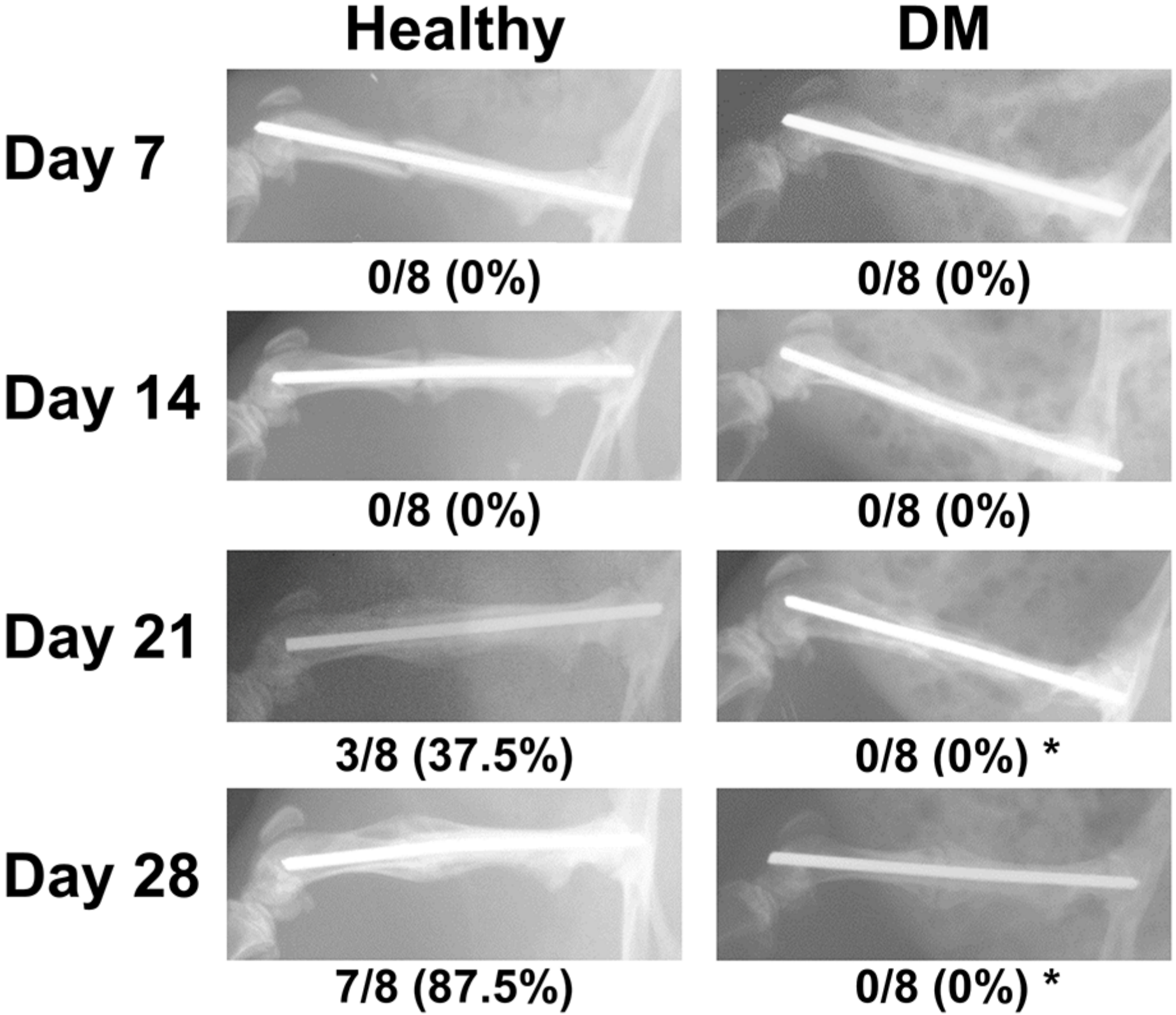
Fig. 1 Radiographs of femurs during fracture healing in the control and diabetes mellitus (DM) groups on post-fracture days 7, 14, 21, and 28. Representative radiographs including the fracture site are shown, and the proportion of rats with fracture union is indicated at the lower part of each image. *$p < 0.05$ compared with the indicated group

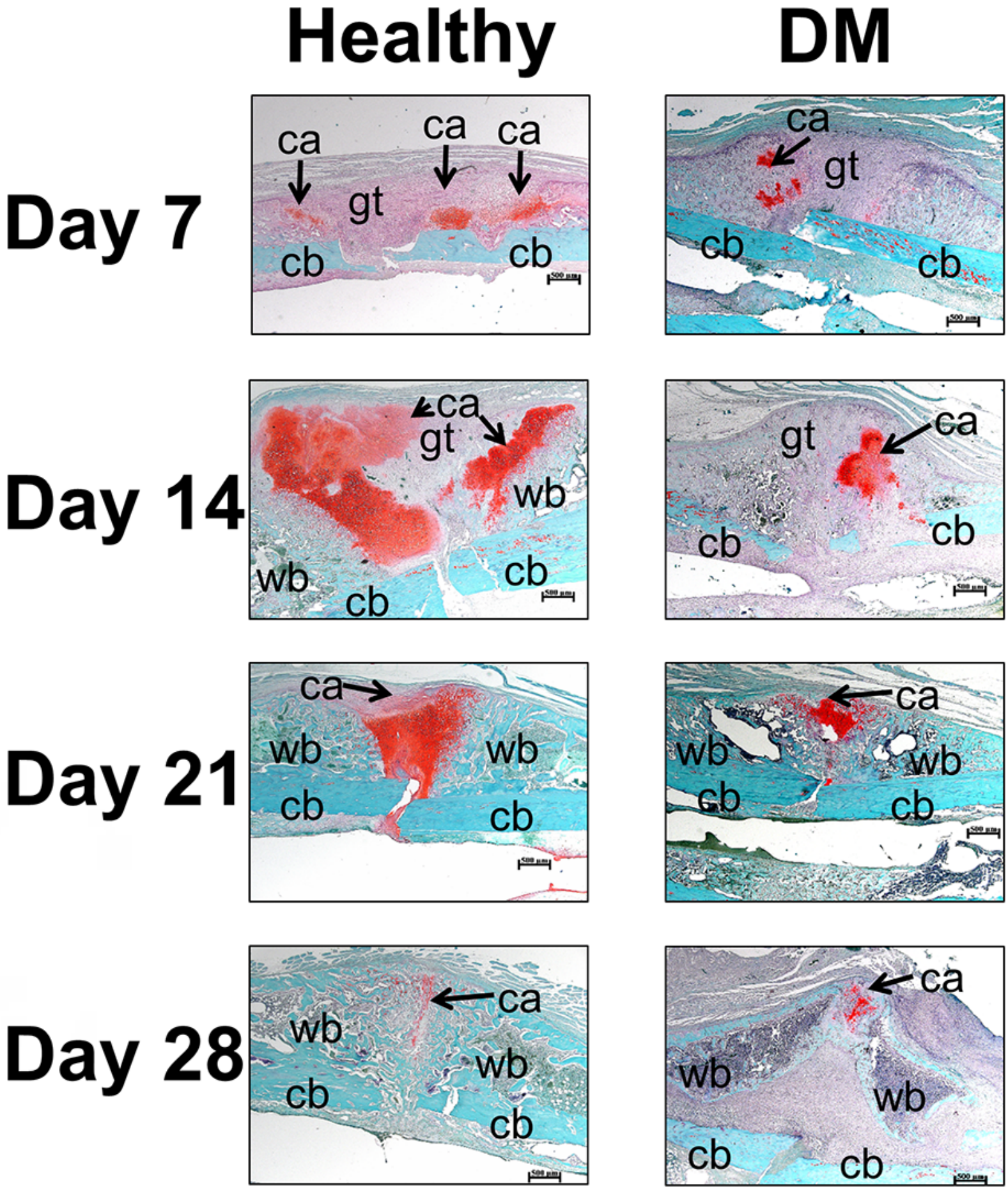
Fig. 2 Histology of fracture sites in the healthy control and diabetes mellitus (DM) groups on post-fracture days 7, 14, 21, and 28. **a** Sections were stained with safranin-O/fast green. Scale bar = 500 μm. cb, cortical bone; ca, cartilage; ft, fibrous tissue; wb, woven bone. **b** Changes in the cartilage area of the DM and healthy groups during fracture healing (n = 5; *$p < 0.05$ within the indicated group)

Fig. 3 Assessment of angiogenesis in the healthy control and diabetes mellitus (DM) groups on post-fracture days 7, 14, 21, and 28. **a** Capillary density is expressed as the average number of capillaries counted in five randomly selected fields (n = 5; *$p < 0.05$ within the indicated group). **b** Representative images of safranin-O/fast green staining in the upper row. Scale bar = 500 μm. ca, cartilage; cb, cortical bone; wb; woven bone. Representative images of fluorescent vascular staining with isolectin B4 (ILB4; green) and 4',6-diamidino-2-phenylindole (DAPI; blue) on day 28 in the lower row. Scale bar = 100 μm. The area surrounded by yellow squares in safranin-O/fast green staining indicates the region of interest observed by vascular staining. Capillaries are indicated in green

Fig. 4 Quantitative real-time PCR analysis of SDF-1 **(a)** and CXCR4 **(b)**. The mRNA levels were

354 normalized to GAPDH and are presented as the fold change (*$p < 0.05$ within the indicated group)
355 relative to levels in the post-fracture day 7 healthy sample (value set at 1 for each marker)
356
357 **Fig. 5** Immunohistochemistry for the callus of fracture sites in the healthy control and diabetes
358 mellitus (DM) groups on post-fracture days 21 (**a**) and 28 (**b**) with the following antibodies: anti-
359 SDF-1 and anti-CXCR4. pc, proliferating chondrocytes; hc, hypertrophic chondrocytes; ob,
360 osteoblasts. Scale bar = 100 μm


Healthy
DM
Day 7
0/8 (0%)
0/8 (0%)
Day 14
0/8 (0%)
0/8 (0%)
Day 21
3/8 (37.5%)
0/8 (0%) *
Day 28
7/8 (87.5%)
0/8 (0%) *


Healthy
DM
Day 7
Day 14
Day 21
Day 28
ca
gt
cb
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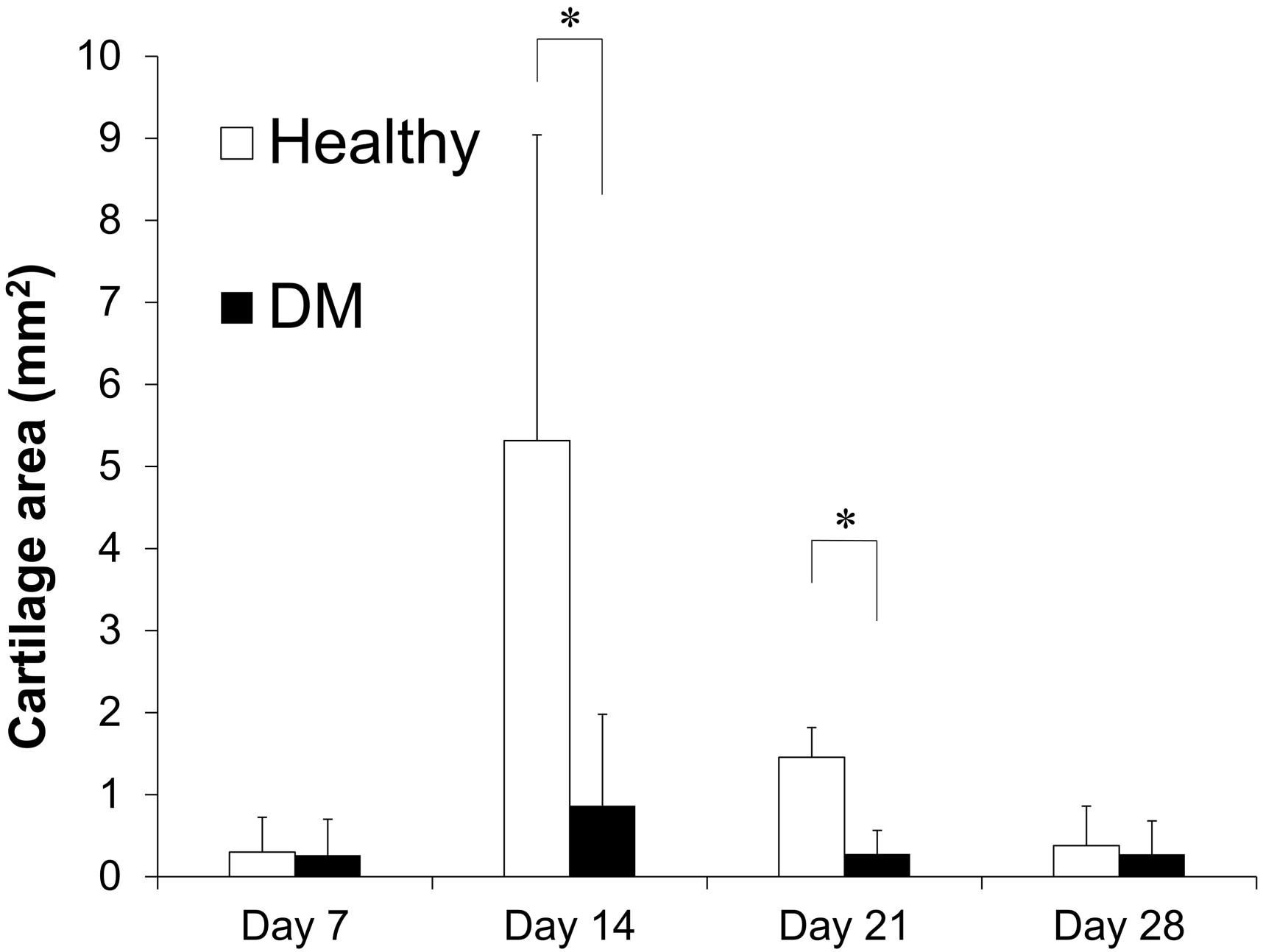
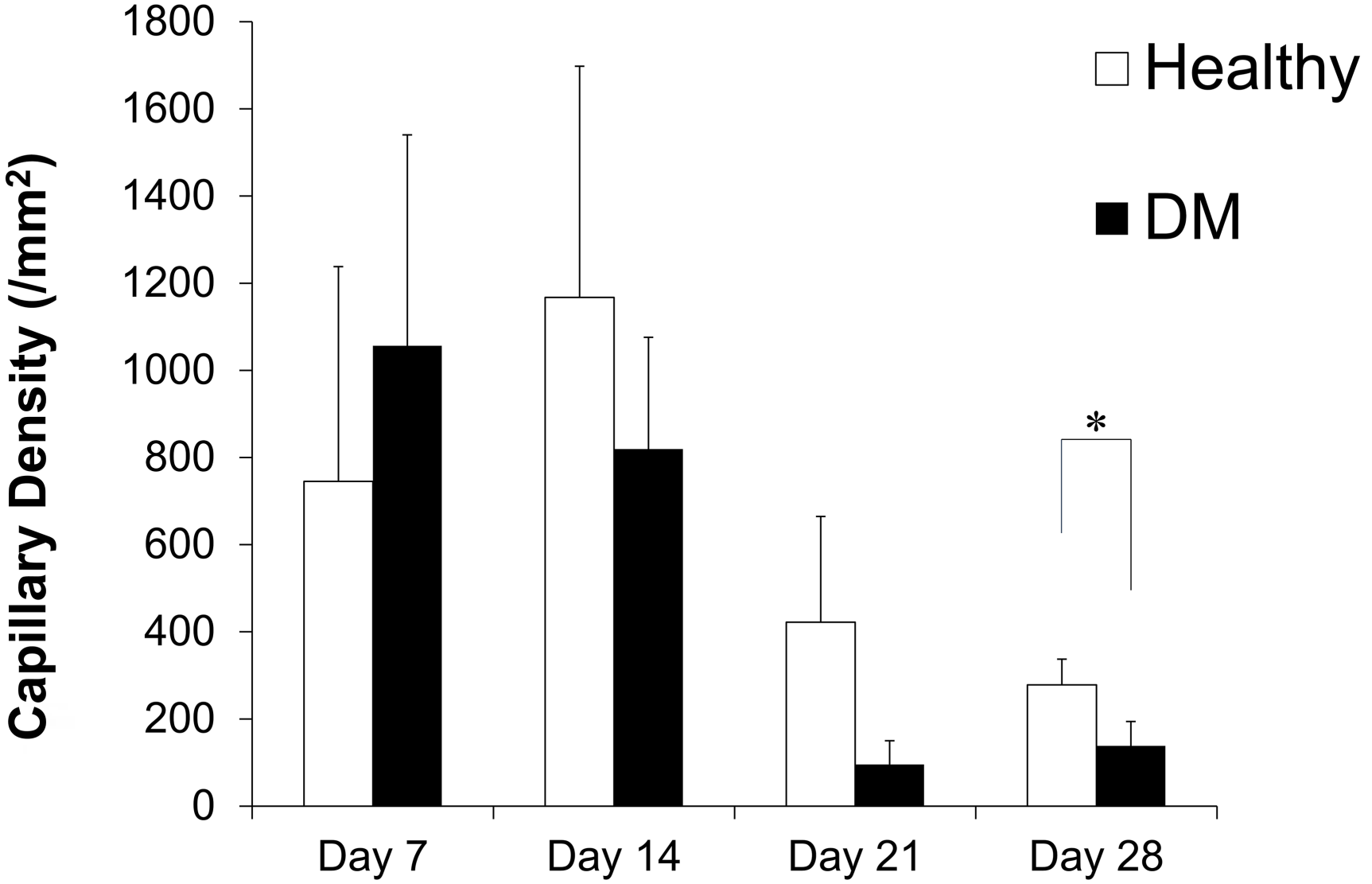

Healthy
DM
Cartilage area (mm²)
10
9
8
7
6
5
4
3
2
1
0
*
*
Day 7
Day 14
Day 21
Day 28

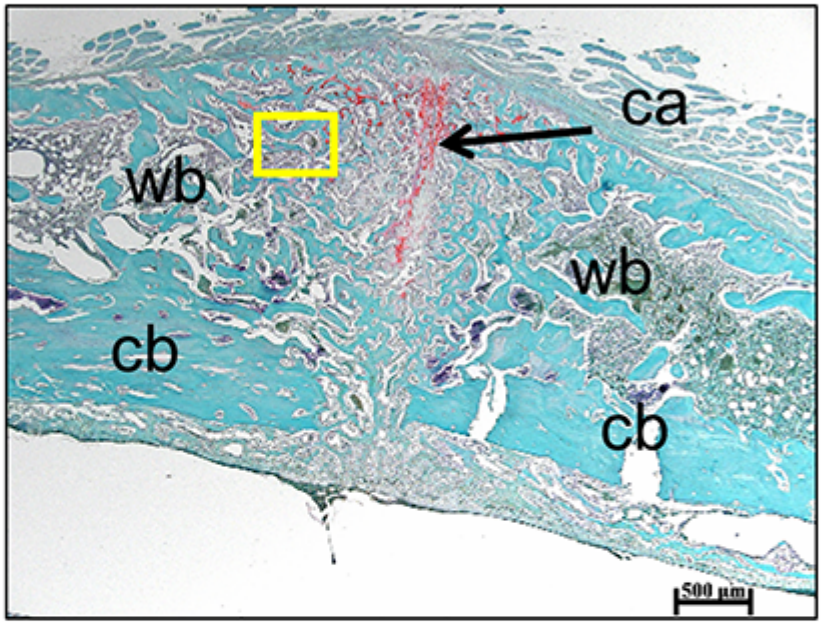

Capillary Density (/mm²)
1800
1600
1400
1200
1000
800
600
400
200
0
Healthy
DM
*
Day 7
Day 14
Day 21
Day 28

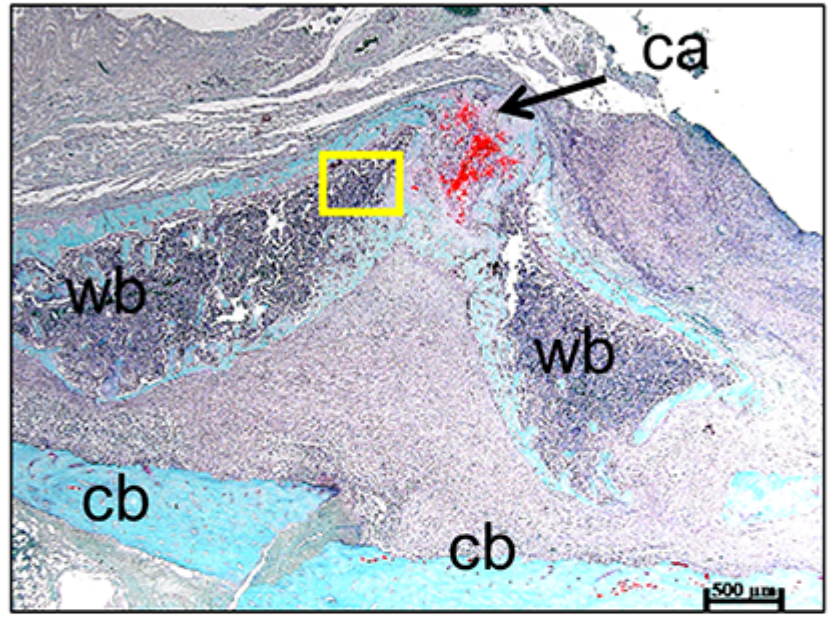
Day 28

Healthy

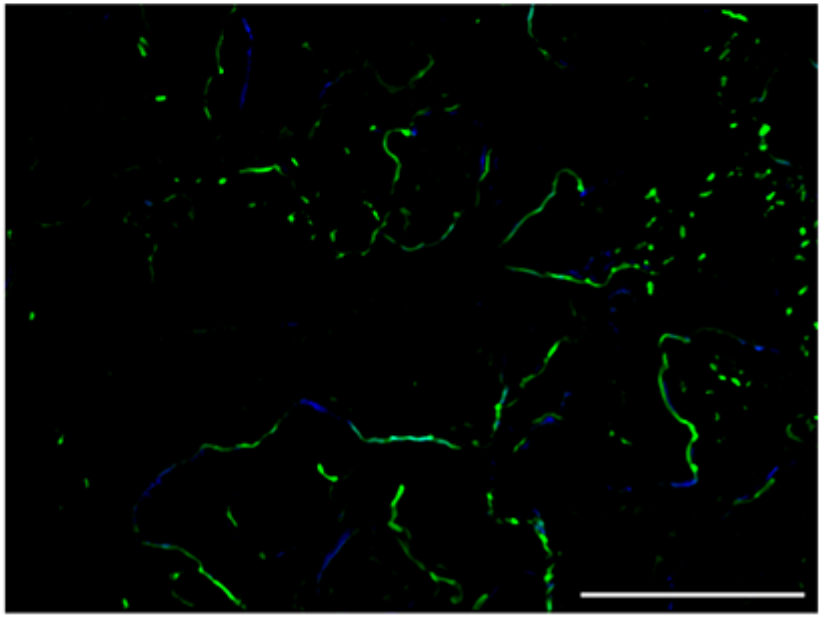
DM

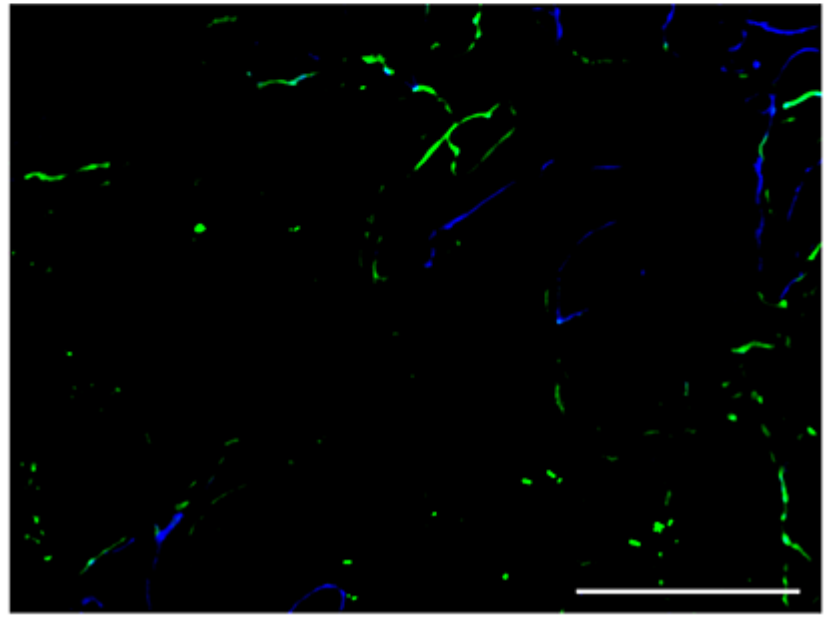
Safranin-O

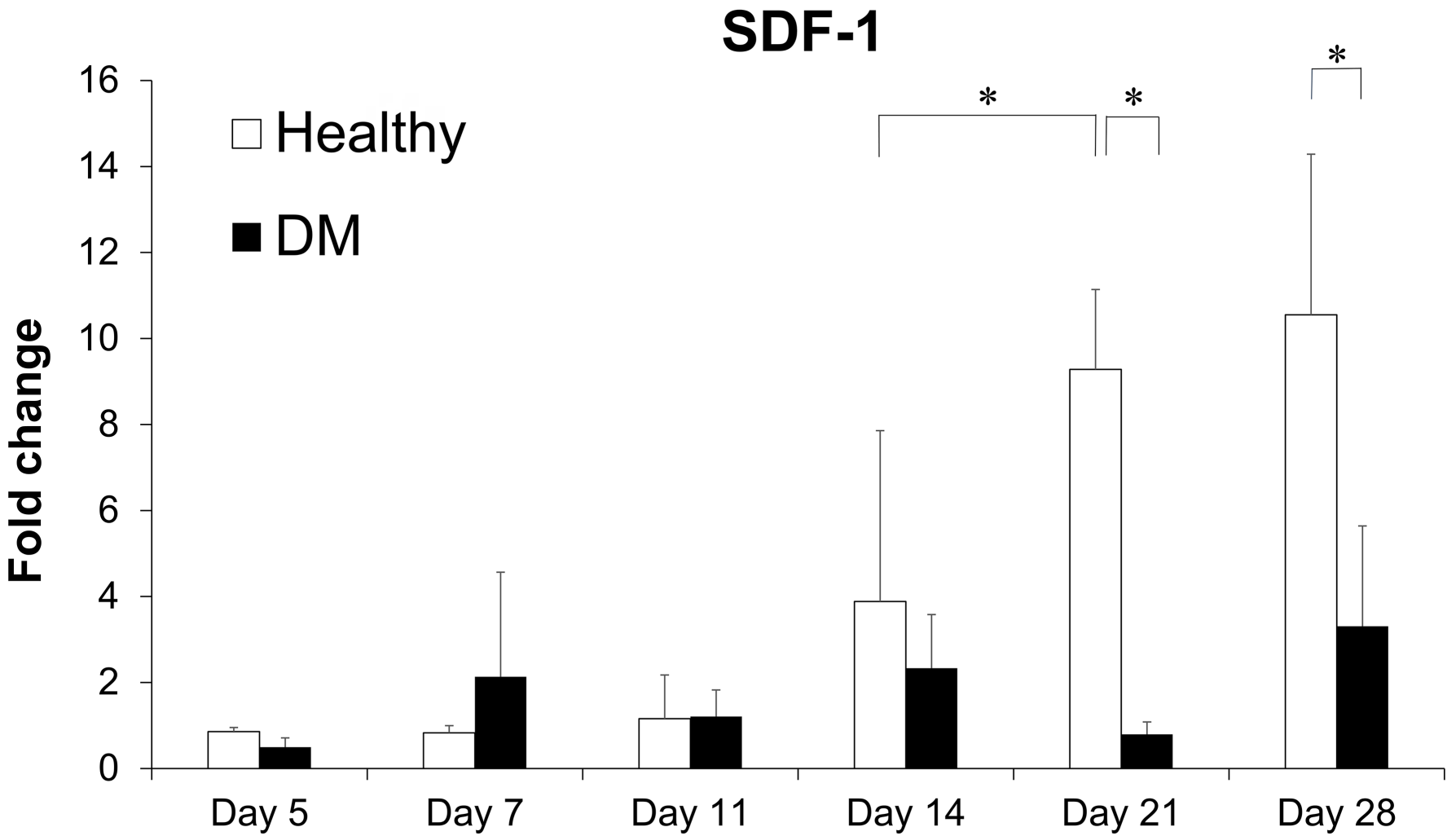


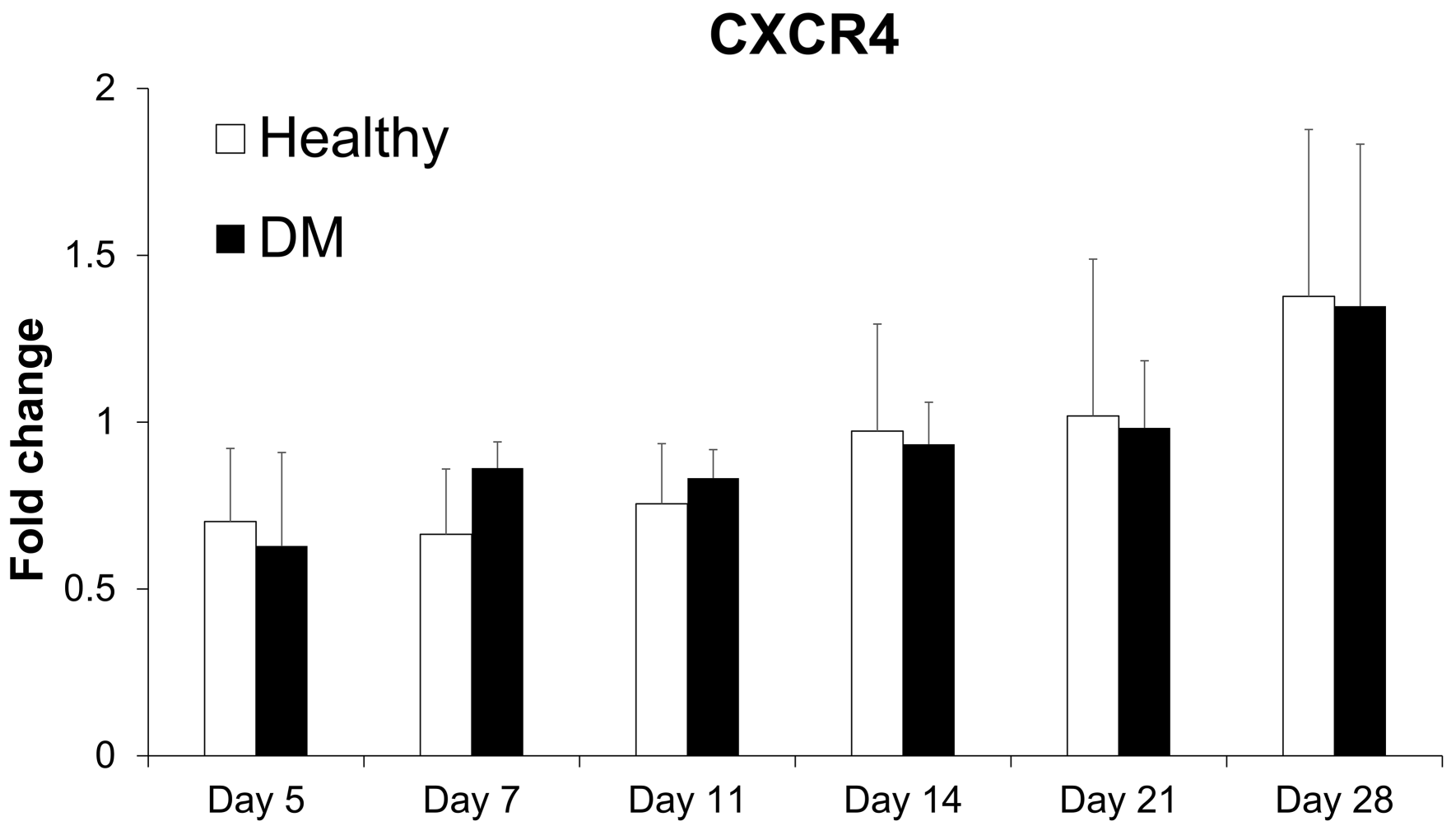


ILB4 + DAPI






SDF-1
Healthy
DM
Fold change
16
14
12
10
8
6
4
2
0
*
*
*
Day 5
Day 7
Day 11
Day 14
Day 21
Day 28

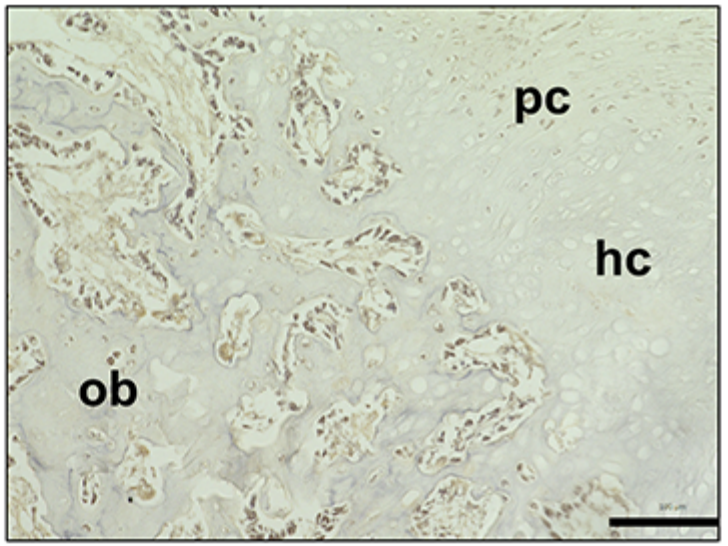

CXCR4
Healthy
DM
Fold change
2
1.5
1
0.5
0
Day 5
Day 7
Day 11
Day 14
Day 21
Day 28

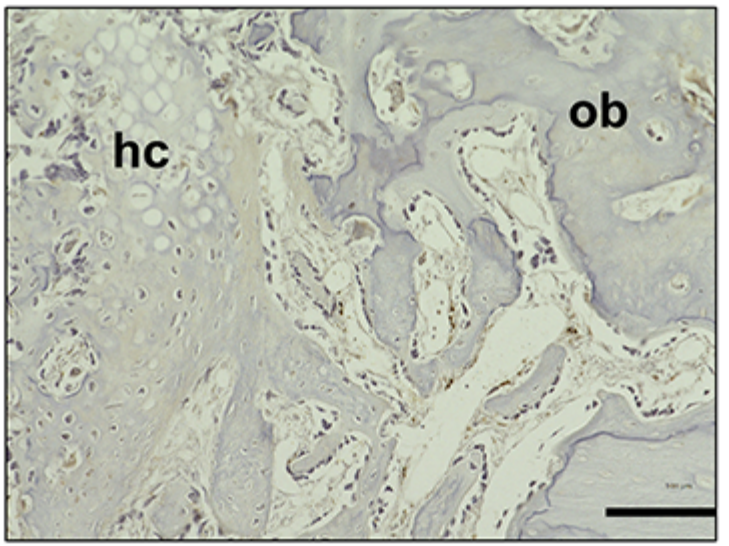
Day 21

Healthy

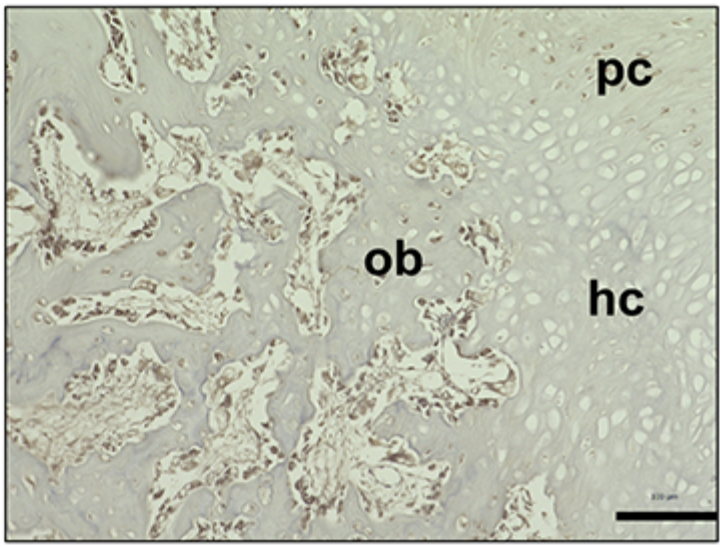
DM

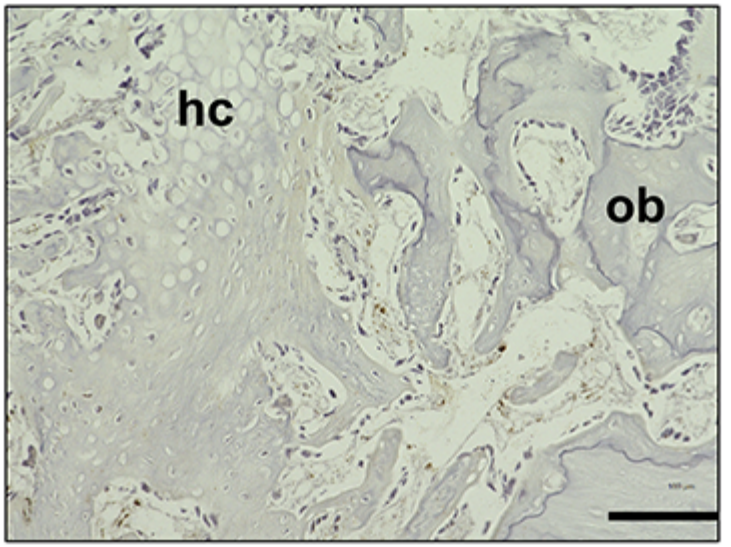
SDF-1





CXCR4





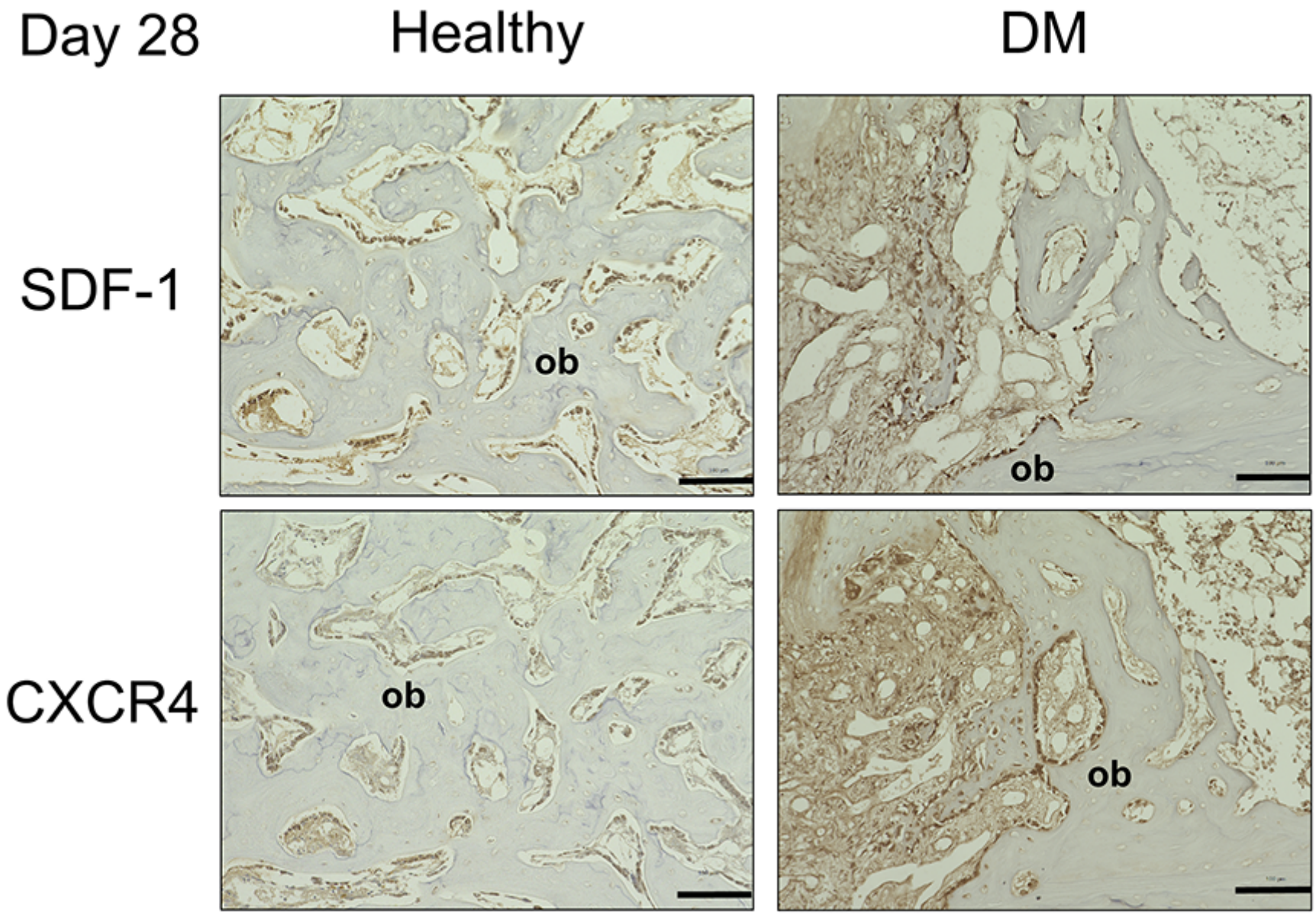

Day 28
Healthy
DM
SDF-1
CXCR4
ob
ob
ob
ob

Table 1 Specific primers used for real-time PCR amplifications

Gene name	Primer sequence (5′-3′)	
	Forward	Reverse
GAPDH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG
SDF-1	GCTCTGCATCAGTGACGGTAAG	TGGCGACATGGCTCTCAAA
CXCR4	ATCATCTCCAAGCTGTCACACTCC	GTGATGGAGATCCACTTGTGCAC

GAPDH glyceraldehyde-3-phosphate-dehydrogenase; SDF-1, stromal cell-derived factor 1; CXCR4, CXC chemokine receptor 4

Table 2 Summary of the immunolocalization patterns of SDF-1 and CXCR4

	Day 21		Day 28	
	SDF-1	CXCR4	SDF-1	CXCR4
Healthy group				
Proliferating chondrocytes	+	+	Not present	Not present
Periosteal cells	+	+	+	+
Osteoblasts	+	+	+	+
DM group				
Proliferating chondrocytes	-	-	+	+
Periosteal cells	-	-	+	+
Osteoblasts	-	-	+	+

SDF-1, stromal cell-derived factor 1; CXCR4, CXC chemokine receptor 4; DM, diabetes mellitus

-, no staining; +, positive staining