Histological Demonstration of Bone Healing in Rat Tibiae Influenced by Diode Laser Irradiation

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半導体レーザー照射によるラット脛骨の骨治癒における組織学的実験

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Summary

Acceleration of the bone healing period is important in clinical situations such as implant and periodontal treatments. Recently, it has been demonstrated that mechanical stimuli including ultrasound and microwaves are potentially powerful treatments for bone regeneration and the acceleration of bone healing. Furthermore, laser irradiation has been shown to stimulate bone formation. However, there are few reports describing the histological changes in bone following laser irradiation. The aim of this study was to examine the effect of diode laser (910 nm) irradiation on histological changes during bone healing in rats. Two groups of rats with bone defects in the tibiae were subjected to laser irradiation at 0, 40, 80, and 120 J; group 1 was irradiated daily with each dose for a total of 14 days, and group 2 was irradiated at 120 J for 7 days and subsequently evaluated for up to 14 days post-irradiation. Tibiae were removed at 3, 7, 14, and 21 days, and subjected to serial sectioning. Morphological examination of bone formation was conducted using hematoxylin-eosin staining and calcein labeling of sections. In group 1, bone formation was stimulated by laser irradiation, and at day 7 the effect was found to be dose-dependent. However, at day 14, bone volume was decreased in a dose-dependent manner. In group 2, the laser-irradiated tibiae showed a greater volume of bone formation than that of the control on day 14. However, no differences in bone volume were observed in the treatment groups on day 21. These results indicated that diode laser irradiation induced marked bone formation in the early phase of the bone healing process and the effects depended on the irradiation dose; however, a longer period of high-power laser irradiation reduced bone formation. This study suggests that diode lasers can be utilized for bone regeneration, taking into consideration the irradiation period and dose.

Key words: diode laser, LLLT, bone regeneration, tibiae, rats

和 文 要 約

骨の治癒時間を短縮することは，インプラントや歯周治療などにおいて重要である。近年では，超音波およびマイクロ波などの物理的刺激が，骨の治癒を促進させることや骨再生のための強力な治療機器となることが実証されている。さらに，レーザー照射においても骨形成を刺激することが報告されている。しかしながら，レーザー照射により変化した骨の組織学的考察を報告する研究は多くない。本研究の目的は，半導体レーザー（910nm）を照射し，ラット脛骨の治癒における組織学的変化を調べることである。レーザー照射は，出力を 0J，40J，80J，および 120J とし，ラット脛骨骨欠損部位にレーザー照射を行い，実験群を 2 群に分けた。実験群 1 は，各エネルギーで照射を 14 日目まで毎日行い，実験群 2 は 21 日間の実験期間中に 120J の出力で照射を 7 日目まで継続し，その後は照射を行わなかった。脛骨は 3，7，14，21 日目で摘出し，連続切片とした。骨形成の骨形態計測は，H-E 染色およびカルセイン標識切片上で実施した。 実験 1 において，7 日目の骨形成はレーザー照射により刺激され，さらにその効果はレーザーの出力に依存していた。しかし 14 日目では，骨量はレーザーの出力に反比例して減少していた。 実験 2 において，7 日目までレーザーの照射（120J）を行った脛骨は，骨修復の促進が観察され，レーザー照射された脛骨における骨形成量は，14 日目の対照群と比較して有意に多かった。しかしながら，21 日目になると，レーザー照射群と対照群間における脛骨の骨量に差はみられなかった。これらの結果より，半導体レーザー照射は骨修復過程の初期段階において骨形成を強く誘導し，そして照射出力に依存していることが示唆された。しかし，骨組織への長期間の過度なレーザー照

射は新生骨量を減少させた。半導体レーザーが骨再生に利用可能であるが，レーザーの照射時間と照射出力に対する考慮が重要であると考えられる。

キーワード：半導体レーザー,LLLT,骨再生,脛骨,ラット

Introduction

In recent years, dental lasers have been used to treat a variety of dental diseases, and the widespread use of lasers in dentistry is growing steadily. The clinical applications of dental lasers are classified into the following two methods: high power laser irradiation (HLLT: high reactive level of laser therapy) used to ablate tissues, and low power laser irradiation (LLLT: low reactive level of laser therapy) used to alter cellular function[1]. It is especially noteworthy that LLLT can be applied for regeneration therapy of several tissues[2].

Diode lasers have wavelengths in the near infrared range (700 – 900 nm) and can penetrate to a depth of 2 to 3 cm depending on the target tissue[3]. For these reasons, diode lasers can be used for tenporo-mandibular disorder pain relief [4, 5], promoting wound healing[6, 7], cell growth[8, 9] as well as soft tissue ablation with high reactive level of laser therapy[1]. Although diode lasers have been beneficial for dental treatment in a variety of clinical situations, there is a paucity of studies reporting on the cellular mechanisms following LLLT irradiation.

Bone regeneration therapies are important in the treatment of periodontal disease and peri-implantitis, and many methods using growth factors[10], cytokines[11], and scaffolds[12] have been developed. In addition, mechanical stress including ultrasound[13,14] and ultra microwave[15] can be applied to stimulate bone metabolism. This is supported by Wolff's law[16] and Frost's mechanostat theory[17], which indicate that bone metabolism reacts to mechanical stress. Given that laser irradiation is a potential mechanical stimulus, it has been postulated that LLLT with a diode laser can be utilized to stimulate bone regeneration. Accordingly, we examined histological changes during the bone repair process in injured tibiae following LLLT by diode laser, and assessed the applicability of diode laser LLLT for bone regeneration therapy.

Materials and methods

1) Preparation of bone defect models

This study was approved by the Animal Experiment Committee of Ohu University

(2013-58) and Meikai University (A1537). Male Sprague-Dawley rats (10 weeks old and weighing 450 ± 20 g, CLEA Japan, Tokyo, Japan) were used in the study. Bone defect models were prepared under isoflurane (Pizer, Tokyo, Japan) and pentobarbital (Kyoritsu, Tokyo, Japan) (1 μL/g) anesthesia. Skin incisions were made to both the right and left tibiae (Fig. 2A). Bone defects were made using a dental steel bur (φ1mm) at high-speed under a spray of normal saline (Fig. 2B). The animals were kept in rearing house with a 12hr light cycle at 24°C, and were given free access to standard animal pelleted food and water.

2) Laser irradiation conditions

Two diode laser machines (Lumix2, DENTALSTIM) were used in this study (Fig.1). The laser irradiation program (45W peak power of superpulsed 910nm, 30kHz frepuency, Average out put power 250mW) was according to manufacturer's instruction. This is a soft laser and is used only for LLLT. Single daily irradiation sessions were performed using the following power conditions: 40 J (2.5 min), 80 J (5 min), and 120 J (7.5 min). The experimental animals were divided into two groups according to the irradiation schedule (groups 1 and 2, n=5 in each group) as follows. In group 1, after the operation, the tibiae were irradiated daily over the skin with each energy condition for 3, 7, and 14 days. In group 2, after the operation, tibiae were irradiated at 120 J/day for the first 7 days. Irradiation was discontinued and the animals were evaluated at 7, 14 and 21 days. Control rats underwent the tibial injury operation but were not subjected to laser irradiation.

3) Preparation of tissue sections and staining methods

Tibiae were fixed with 10% formalin neutral buffer solution (Wako Pure Chemicals, Osaka, Japan) for 3 days at 4°C, and the tissues were cut into small pieces (about 1 cm^3). Then the tissues were decalcified with 10% EDTA for 4 weeks at 4°C. The tibiae were embedded in paraffin and serially sectioned using a microtome (4μm). Sections were stained with hematoxylin-eosin (H-E) for histological analysis. Bone histomorphometry was used to assess bone formation; double labeling was performed with subcutaneous

injections of 10 mg/kg body weight of calcein (Wako Pure Chemicals, Osaka, Japan) 7 and 3 days before sacrifice of animals. The calcein-labeled tibiae were embedded in resin (Technovit 8100; Heraeus KILZER, GmbH, Germany) and serially sectioned (2μm).

4) Bone histomorphometry

H-E sections and calcein labeling sections were observed using a microscope (×40). Images were captured using a digital camera (BX41; Olympus, Tokyo, Japan) and used for bone histomorphometry. Histomorphometry was performed using ImageJ image analysis software (NIH, Bethesda, MD, USA). Trabecular bone formation was measured at the injured regions of tibiae to determine the new bone area per unit tissue area (Bone Area/Tissue Area: BA/TA %) using H-E stained sections. Mineral apposition rate (MAR, μm/day) of newly formed trabecular bone was measured at 10 sites randomly as the width of the gap in calcein-labeled bone (Fig.3). Results are expressed as the means ± SEM, and were evaluated using the Mann-Whitney *U*-test. A P value of less than 0.05 was considered to be significant.

Results

Temporal changes in bone formation of tibiae with and without laser irradiation

A) Group 1

H-E stained sections showing bone formation in rat tibiae are presented in Fig.4, 5, 6. On day 3 after the operation, no bone formation was observed in control sections (Fig. 4a) and laser irradiated tibiae (Fig. 4b: 40 J, Fig. 4c: 80 J, and Fig. 4d: 120 J). On day 7 after the operation, bone formation of laser irradiated tibiae was 5-9% higher than that of control (Fig. 7). Laser irradiation stimulated bone formation in a dose-dependent manner (Fig. 5a, b, c, d, and 7). Laser irradiation at 120 J most strongly induced bone formation of all doses (Fig. 5d and 7). There were significant differences of bone volume (BA/TA) between laser irradiation and control groups ($P<0.05$, $P<0.01$). In contrast, laser irradiation for 14 days reduced bone formation about 3-5% (Fig. 7), and

the bone formation in tibiae at all irradiation doses was less than that of the control group (Fig. 6a, b, c, d, and 7). There were significant differences of bone volume (BA/TA) between laser irradiation and control groups (P<0.05).

B) Group 2

The histological features of bone formation in tibiae laser irradiated at 120 J for 7 days and assessed at 14 and 21 days are presented in Figs. 8 and 9. The tibiae were laser irradiated at 120 J for 7 days and subsequently assessed at 14 and 21 days. On day 14, laser irradiated tibiae showed a larger amount of bone formation (7%) than that of the control tibia (Fig. 8a, b, and 9); however, no differences in bone formation were observed between laser irradiated and control tibiae at 21 days (Fig. 8c, d, and 9). Laser irradiated tibiae at 120 J for 7 days showed a 2-fold increase in MAR, but on day 21, the tibiae without laser irradiation for the remaining 14 days showed no significant difference compared to the control tibiae (Fig. 10).

Discussion

Bone regeneration therapy contributes to diverse dental clinical situations, but the healing period has serious effects on the prognosis and therapeutic value. Given that the healing period is shortened, this will be of benefit to patients as well as clinicians. In this study, we investigated the use of diode laser LLLT in bone regeneration, because LLLT has few side effects on organs and is very easy to use. In addition, depending on the wavelength, the diode laser can penetrate to a tissue depth that includes the bone region[3]. Several studies[18-20] have reported the effects of diode laser on bone metabolism; however, the findings regarding irradiation methods and histological changes during bone regeneration have been varied. Our results will contribute to the development of laser therapy for bone regeneration.

Wolff's law[16] indicates that bone tissue can adapt to mechanical use and mechanical stress promotes bone formation and resorption. Thus, it is reasonable to apply mechanical stress including exercise[21], ultrasound[13,14] and ultra microwave[15] to bone

regeneration. In fact, animal experiments[22,23] reported that mechanical stress influenced bone metabolism. We hypothesized that stimulation by laser can mimic mechanical stress in controlling bone metabolism, and we demonstrated the power-dependent effects of the laser on bone formation during the bone repair process. Calcein labeling revealed that diode laser irradiation directly stimulated osteoblast function and increased mineralization. These facts support the potential therapeutic use of the diode laser, and this study showed that LLLT of diode laser could be applied to bone regeneration.

Next, we discuss the cellular mechanism of LLLT that promotes bone formation. Bone tissues contain large amounts of osteocytes in the mineralized matrix, and the cells are connected to each other by dendritic processes, making up the cell network in bone matrix[24,25]. The cells communicate with each other as well as control bone metabolism. Bonewald[24,25] indicated that osteocytes express sclerostin and Dmp-1, and these proteins are key factors in controlling osteoblast and osteoclast functions. Sclerostin[26] is an inhibitor of the Wnt signaling system and inhibits bone formation in osteoblasts, while dmp-1[27] directly stimulates bone formation of osteoblasts. Furthermore, osteocytes respond to mechanical stress by flowing bone fluid through the osteocyte lacunocanalicular system (fluid-flow shear stress)[24,25]. Interestingly, mechanical stress inhibits sclerostin expression but stimulates Dmp-1 expression[22,23,26]. This is thought to be one of mechanisms regulating bone formation induced by mechanical stress. In addition, our colleagues[28,29] demonstrated that low-power laser irradiation reduced *Sost* mRNA expression, but stimulated *Dmp-1* mRNA expression in cultured osteocyte-like cells. These facts indicate that the mechanism of bone formation induced by laser irradiation may be similar to that of mechanical stress. Diode laser LLLT may also act on osteocytes and influence the expression of sclerostin and Dmp-1 in rat tibiae, resulting in stimulation of bone formation. However, our study did not include an osteocyte experiment, and further studies are needed to elucidate this point.

Notably, we observed that high-power irradiation reduced bone formation in a dose-dependent manner. Although many studies[18-20] have reported that diode laser LLLT could induce bone formation, to the best of our knowledge, this is the first study

to report the opposing effects of laser on bone metabolism. Frost's mechanostat theory[17] proposed that the stimulus for bone functional adaptation is strain magnitude. Bone metabolism under an overload of mechanical stress showed increased bone formation; however, excessive mechanical stress induced immature bone tissues. In the present study, continuous irradiation of LLLT for 7 days stimulated bone formation, whereas 14-day irradiation inhibited bone formation and reduced the volume of newly formed bone. However, irradiation conducted for 7 days was capable of maintaining increased bone volume for up to 7 days post-irradiation. Given that the diode laser LLLT stimulus is similar to that of mechanical stress, it has been suggested that 7 day LLLT was suitable for increasing bone formation, whereas 14 days irradiation represented an excessive stimulus. This may result in a decreased volume of bone formation in tibiae irradiated with continuous LLLT for 14 days. Moreover, rat tibiae at 7 days after the operation correspond to the proliferative phase in which bone healing may be very sensitive to laser irradiation, because immature mesenchymal cells differentiate into osteoblasts during the proliferative phase of the bone healing process[30].

Conclusion

The present study indicated that diode laser LLLT strongly promoted bone formation during the bone healing process; however, an excessive period of laser irradiation reduced bone formation. This suggests that diode lasers are potentially useful devices for bone regeneration, but the effects on bone formation depend on the power of laser irradiation and its timing in the bone healing process.

Conflicts of interest

The authors state that they have no conflicts of interest.

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Figure legends

Fig. 1

The diode laser used in this study (Cold laser Lumix 2; DENTALSTIM).

Fig. 2

Generation of bone defects in rat tibia (a) using a dental bur (b).

Fig.3

Calcein labeling section in rat tibiae. Asterisk indicated bone defects. Allows indicated the width of the gap in calcein labeled bone.

Fig. 4

Histological findings in rat tibiae subjected to laser irradiation. Histological sections of tibiae at 3 days (a-d). Tibiae that were not irradiated with laser were used as control (a). Tibiae were laser irradiated at 40 J (b), 80 J (c), 120 J (d). (Scale bar=200μm)

Fig.5

Histological findings in rat tibiae subjected to laser irradiation. Histological sections of tibiae at 7 days (a-d). Tibiae that were not irradiated with laser were used as control (a). Tibiae were laser irradiated at 40 J (b), 80 J (c), 120 J (d). (Scale bar=200μm)

Fig.6

Histological findings in rat tibiae subjected to laser irradiation. Histological sections of tibiae at 14 days (a-d). Tibiae that were not irradiated with laser were used as control (a). Tibiae were laser irradiated at 40 J (b), 80 J (c), 120 J (d). (Scale bar=200μm)

Fig.7

Newly formed trabecular bone volume (BA/TA%) in rat tibiae laser irradiated at each

power for 3, 7, and 14 days. Results are mean ± SEM (n=5). *P<0.05, **P<0.01 compared with the control.

Fig.8

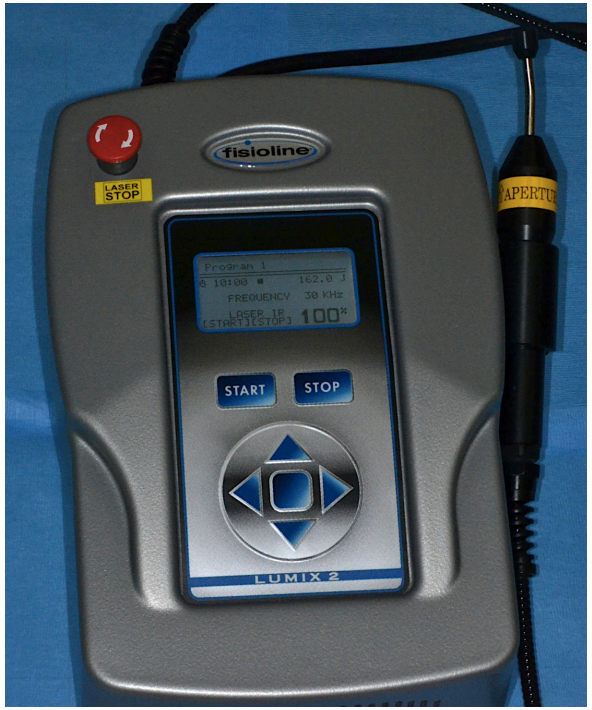
Histological findings in rat tibiae at 14 (a and b) and 21 days (c and d). Tibiae were irradiated at 120 J/day for 7 days only and evaluated at 14 (b) and 21 (d) days. Control tibiae (a and c) were not exposed to irradiation during the experimental period. (Scale bar=200μm)

Fig.9

Newly formed trabecular bone volume (BA/TA%) in rat tibiae laser irradiated at 120 J at 14 and 21 days. Results are mean ± SEM (n=5). *P<0.05 compared with the control.

Fig.10

Mineral apposition rate (MAR) of newly formed trabecular bone in tibiae. Irradiation at 120 J was performed for 7 days and then discontinued for the remaining 7 days of the experimental period (14 days in total). MAR was measured as the width of calcein double labeling lines in newly formed trabecular bones in tibiae. Results are mean ± SEM (n=5). **P<0.01 compared with the control.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LUMIX 2

Fig.1

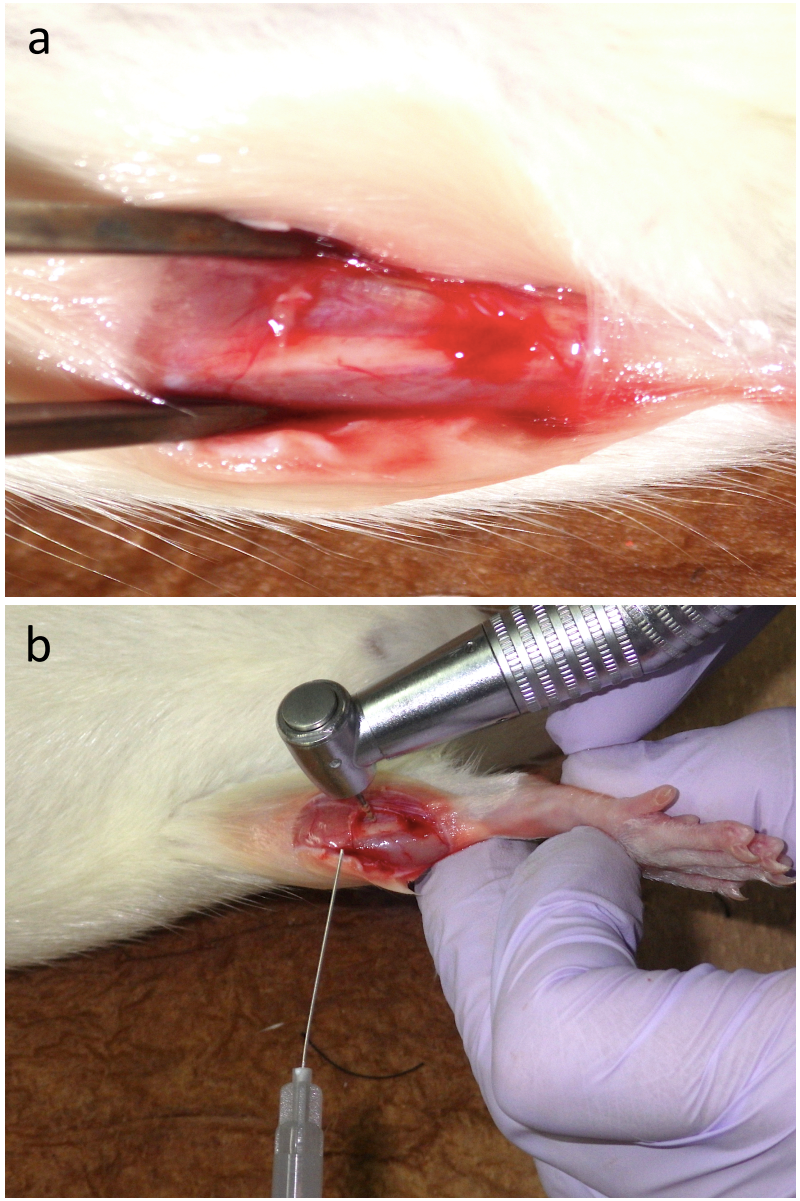

a
b

Fig.2

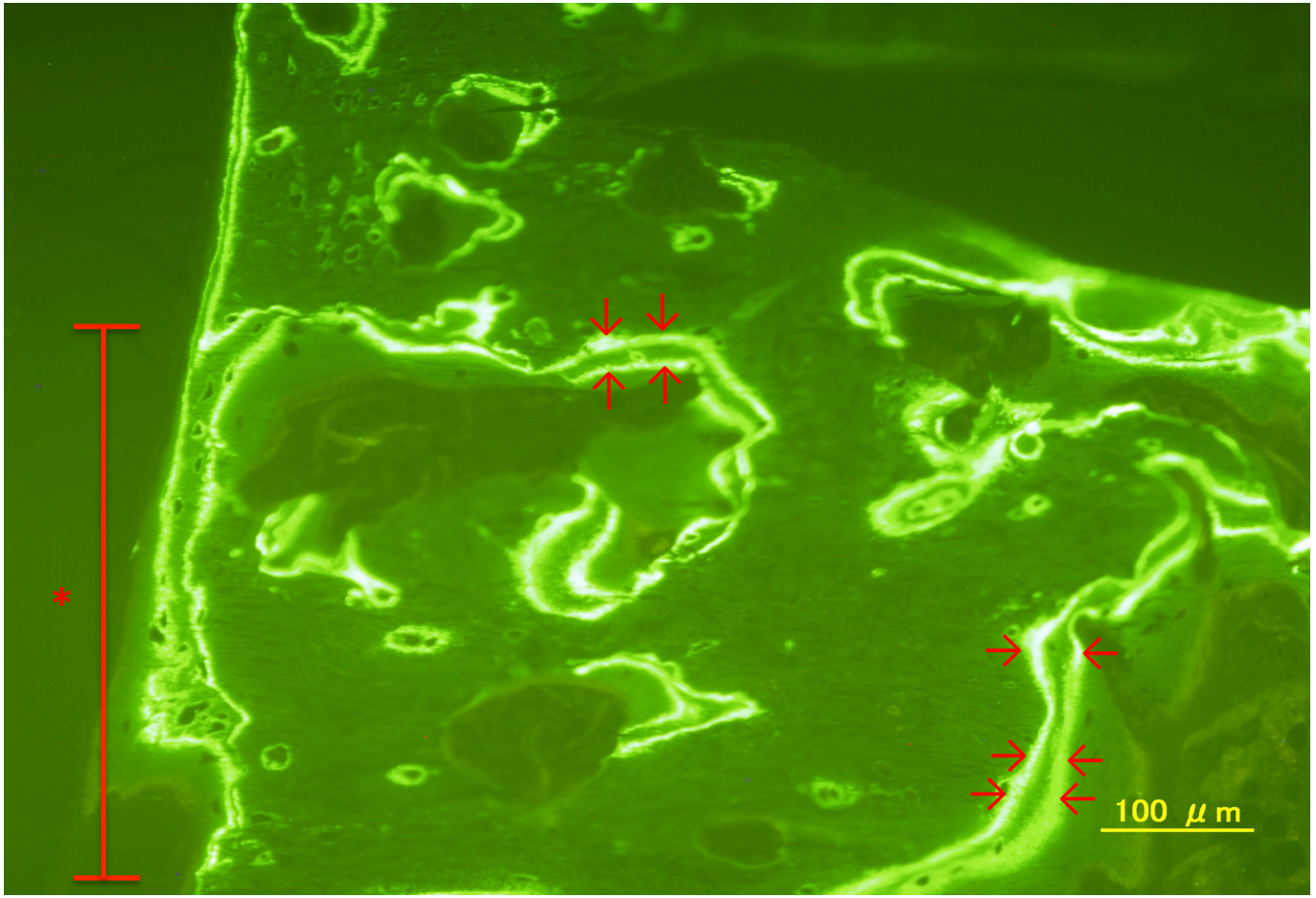


Fig.3

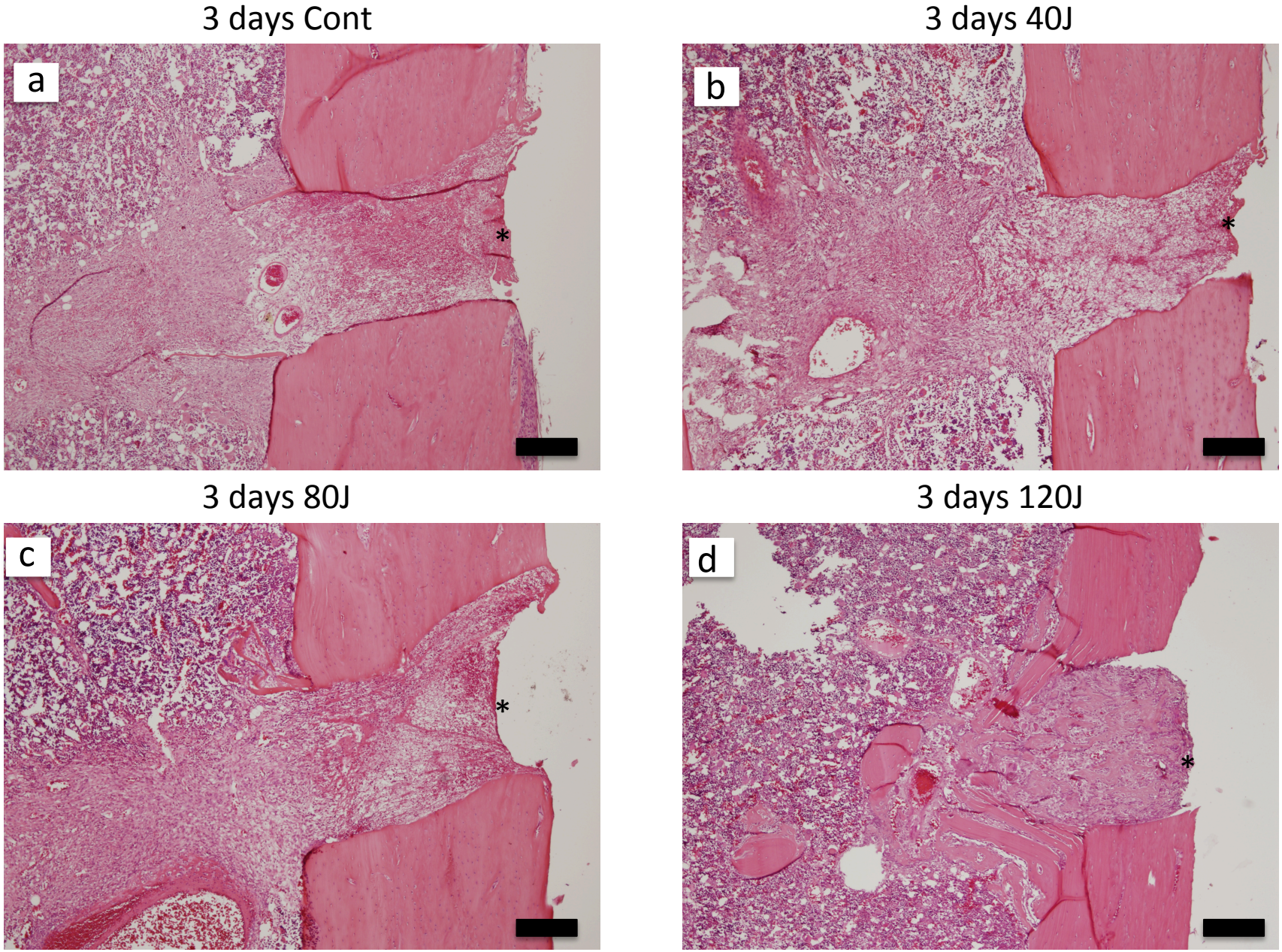
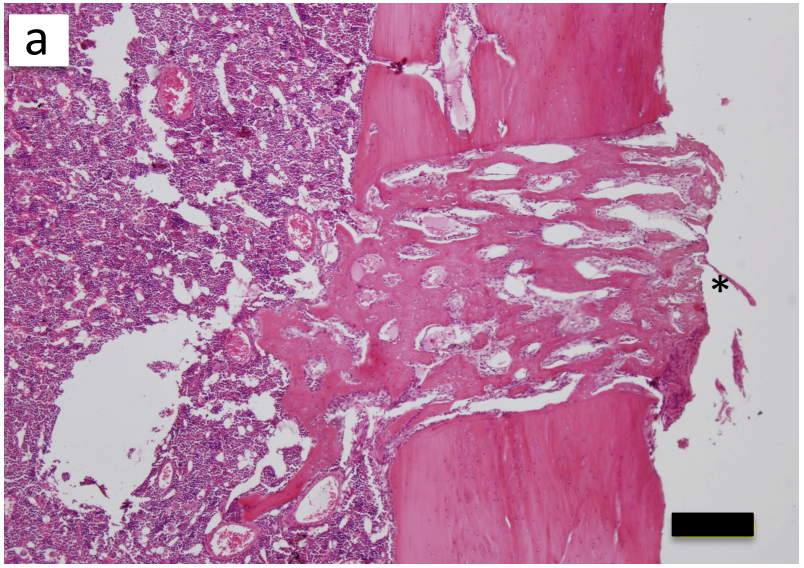
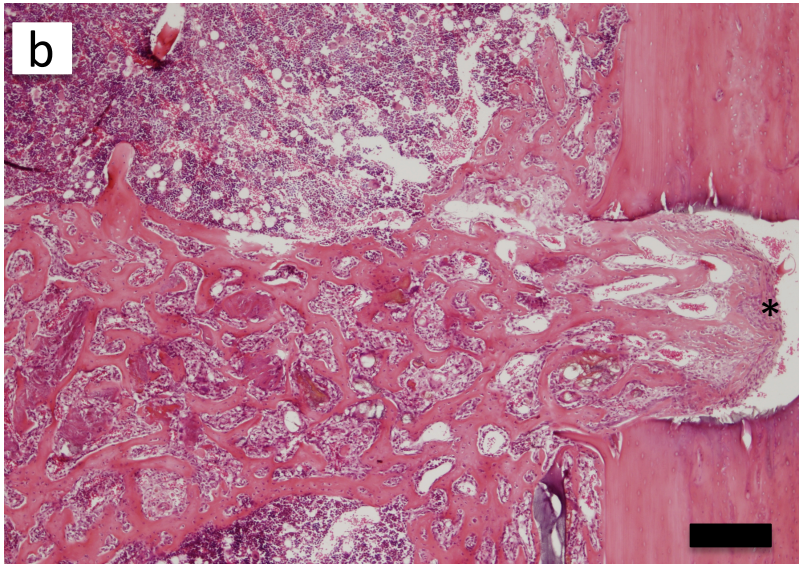


Fig.4

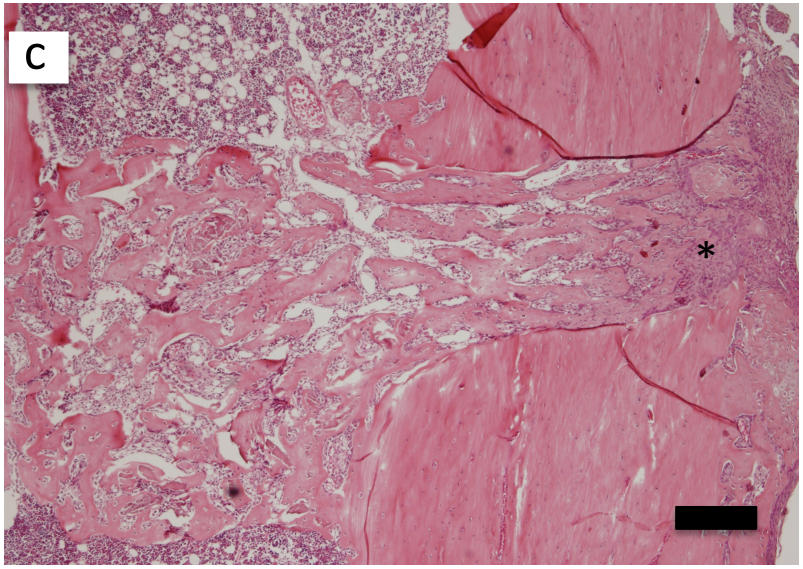
7 days Cont



7 days 40J



7 days 80J



7 days 120J

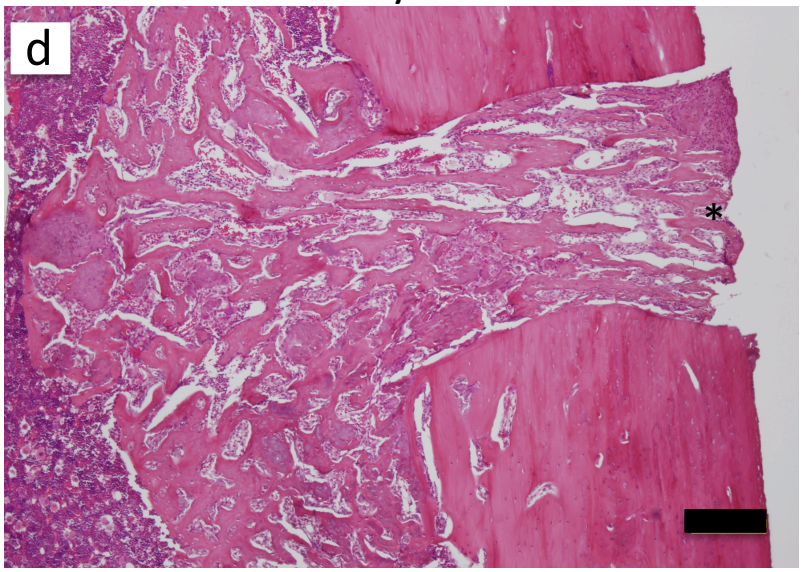


Fig.5

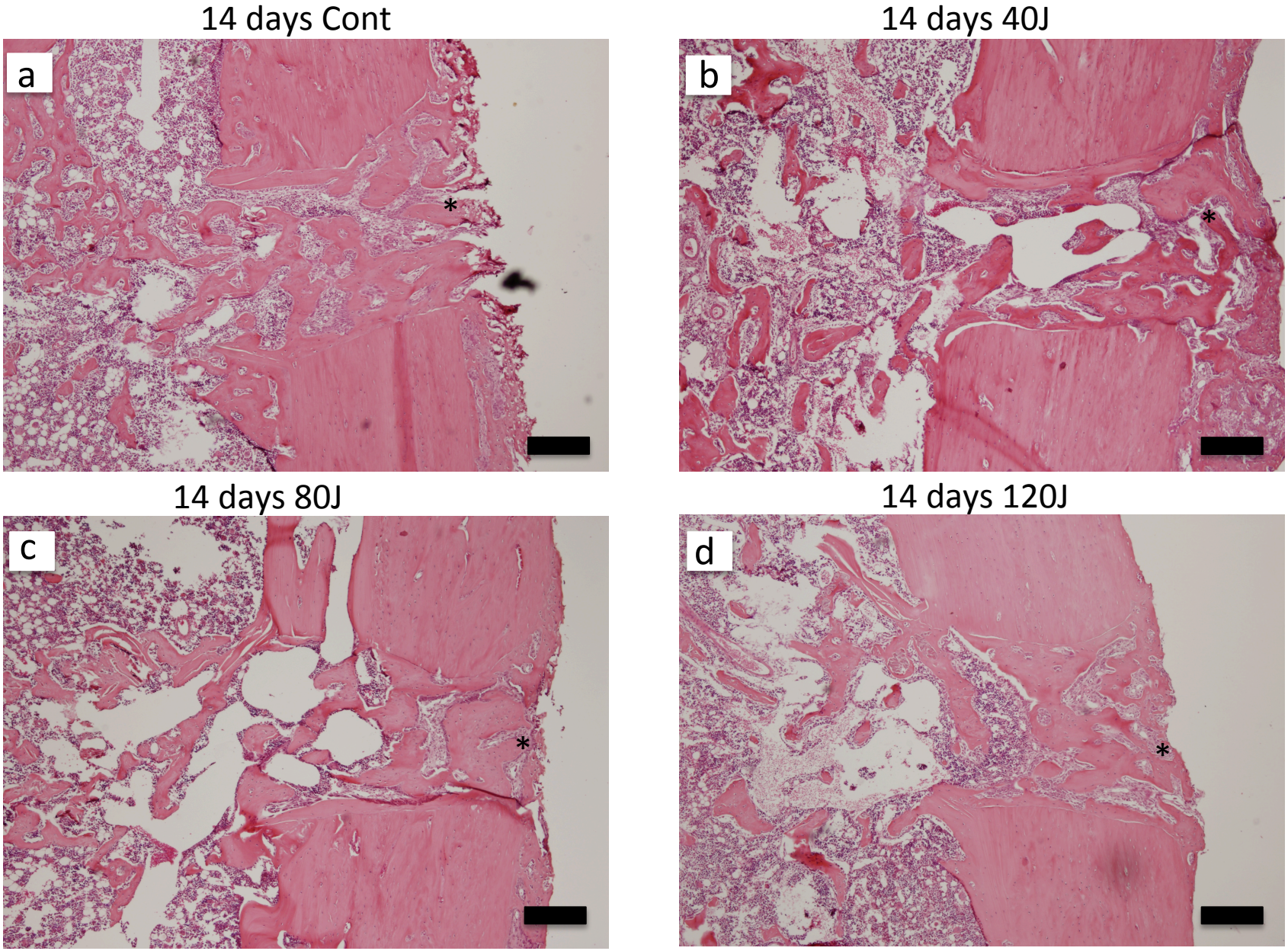
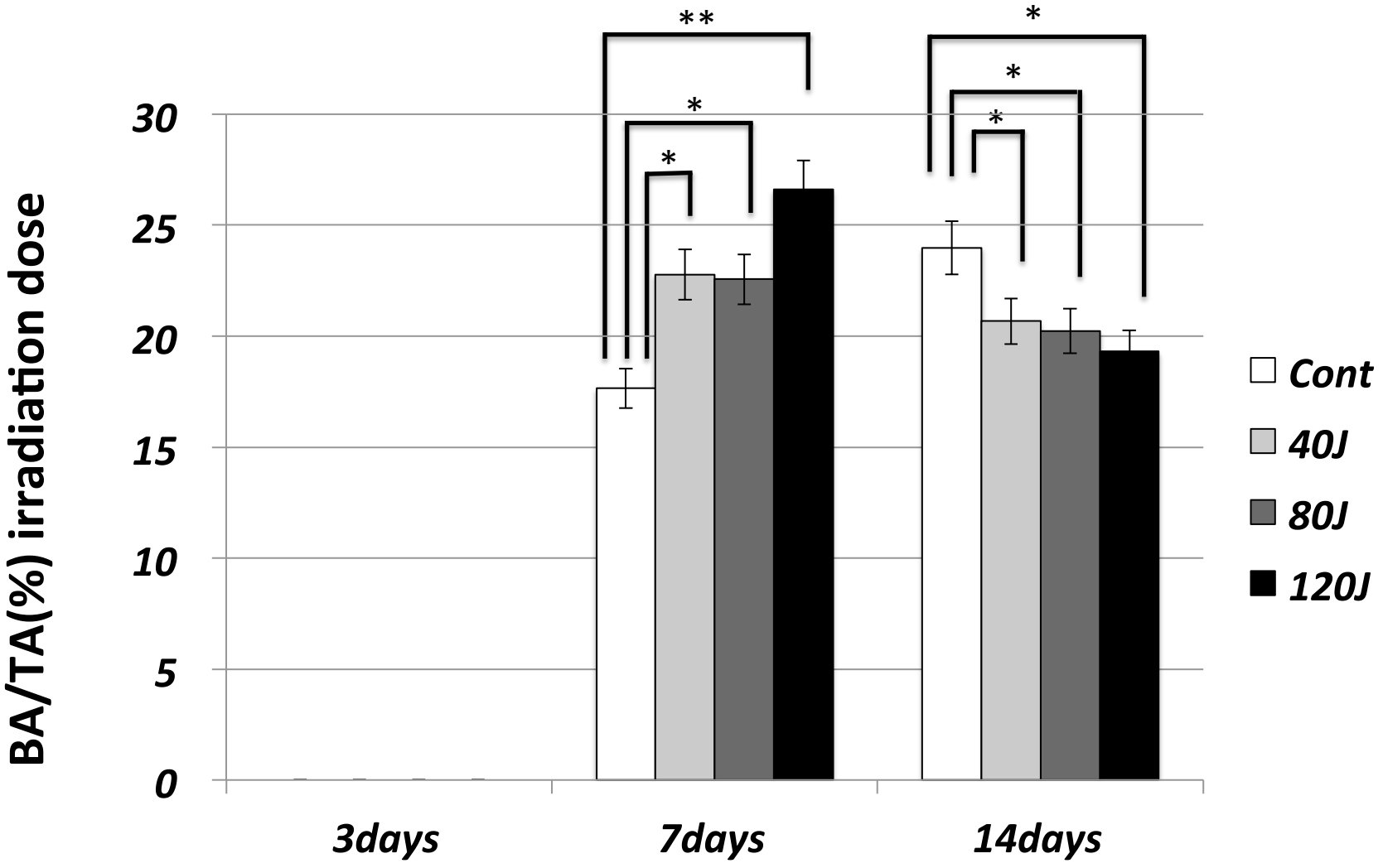


Fig.6



*:p<0.05 **:p<0.01

Fig.7

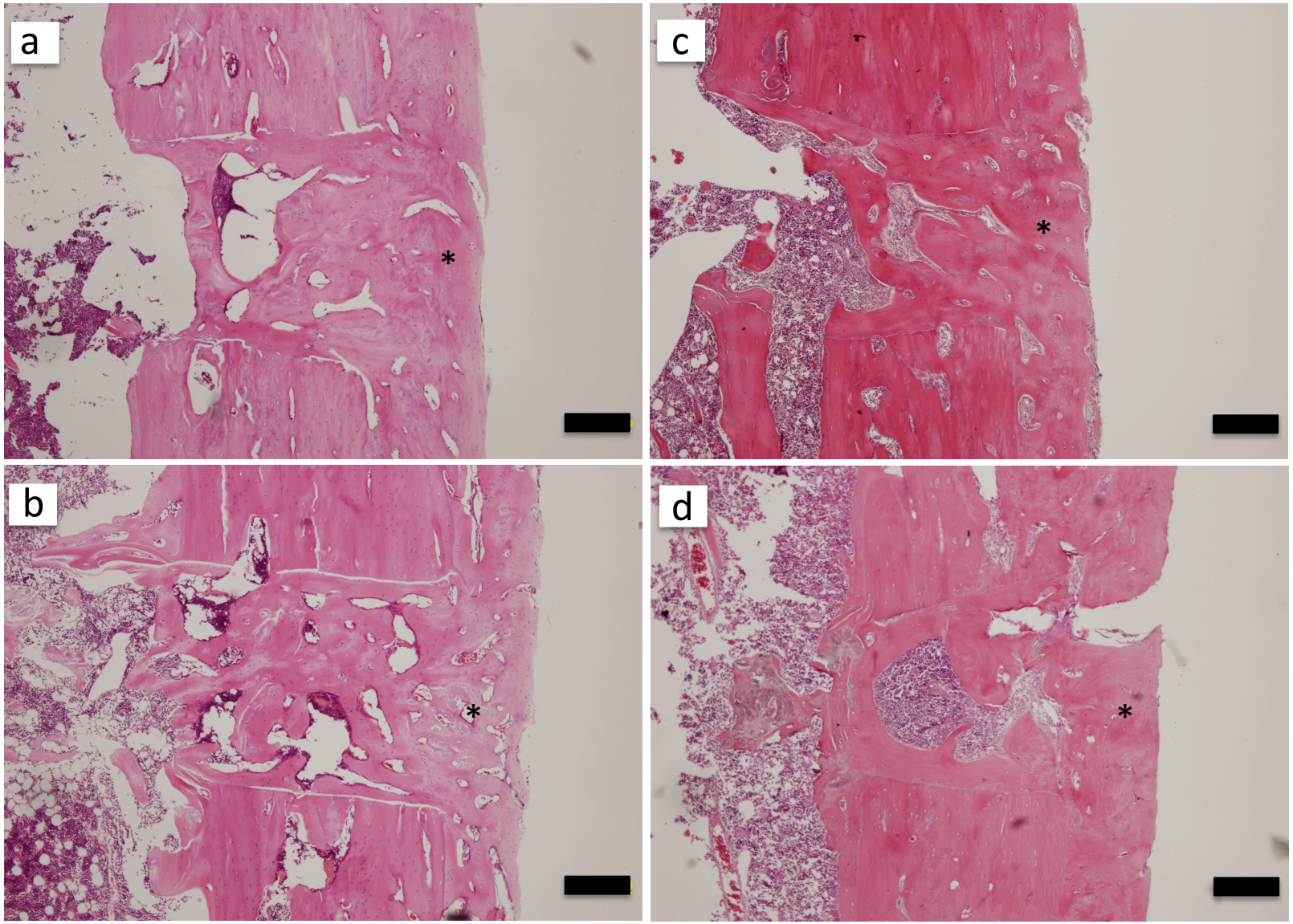


Fig.8

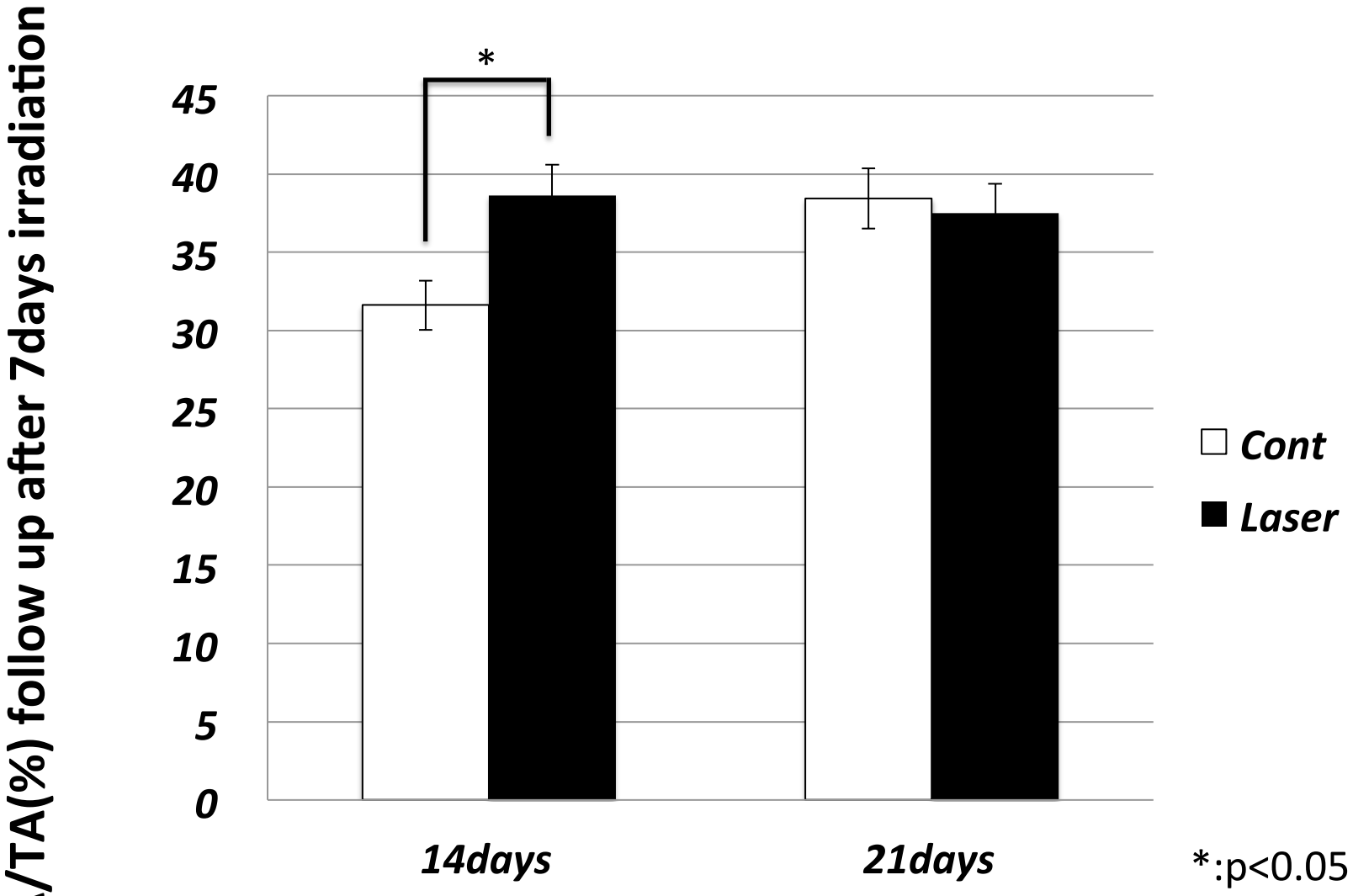

BA/TA(%) follow up after 7days irradiation
*
45
40
35
30
25
20
15
10
5
0
14days
21days
Cont
Laser
*:p<0.05

Fig.9

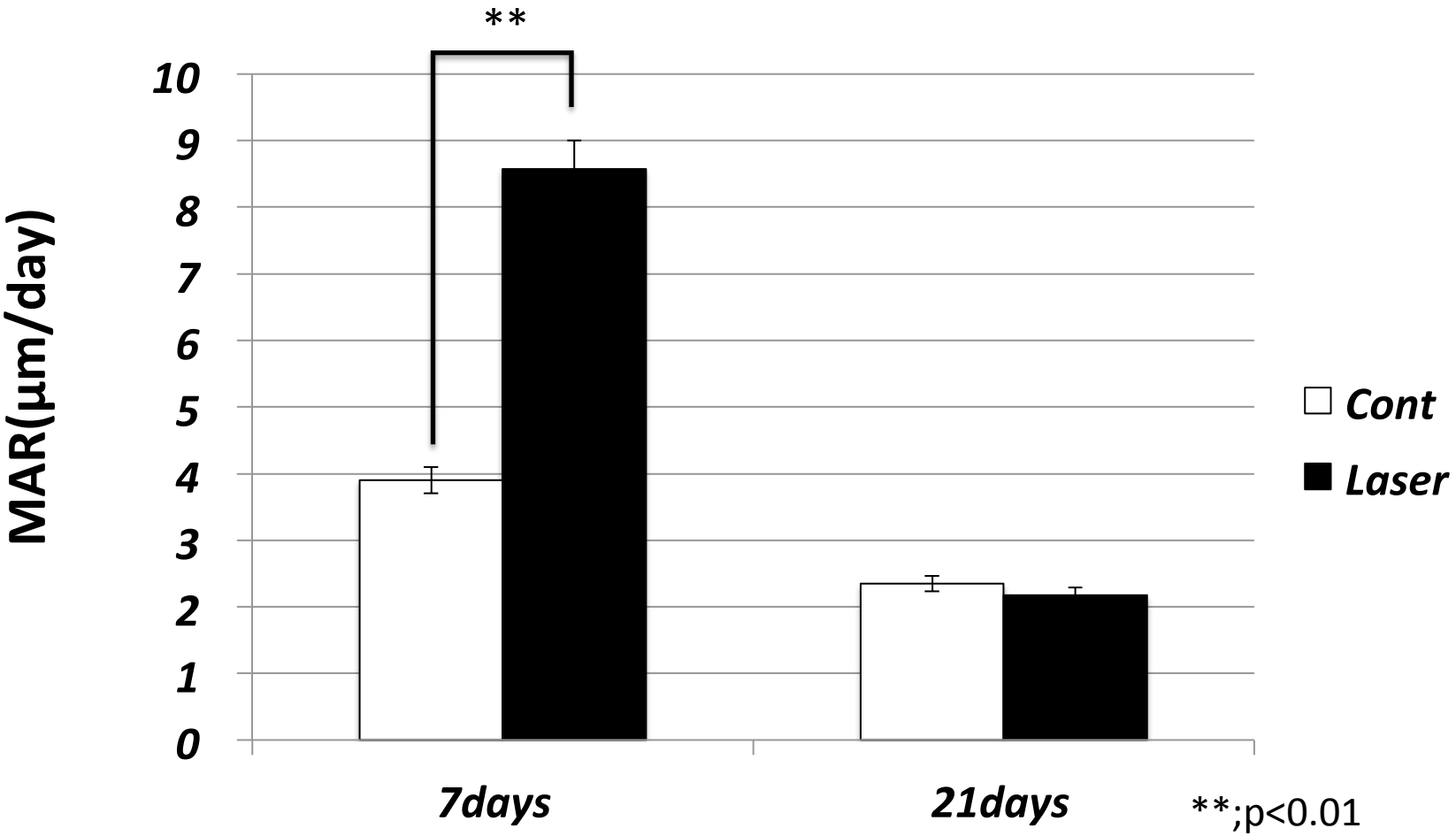


Fig.10