学 位 論 文

Cytological Kinetics of Periodontal Ligament in an Experimental Occlusal Trauma Model

（実験的咬合性外傷による歯根膜の細胞動態）

髙谷　達夫

大学院歯学独立研究科　健康増進口腔科学講座　口腔健康分析学
（主指導教員：藤井　健男　教授）

松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

Cytological Kinetics of Periodontal Ligament in an Experimental Occlusal Trauma Model

Tatsuo Takaya

Department of Oral Health Promotion, Graduate School of Oral Medicine
（Chief Academic Advisor：Professor Takeo Fujii）

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要 旨

【目的】外傷性咬合による歯周組織への影響については、十分な実証的研究が進展していない。そこで咬合性外傷を発症する動物実験モデルの開発により、高度な骨吸収の成因に関与すると考えられる外傷性咬合の歯根膜組織に及ぼす細胞動態を検討した。

【材料と方法】7 週齢の ddY マウス 12 匹および C57BL/6 マウス(GFP 骨髄移植マウス) 8 匹を使用した。腹腔内麻酔を行い、手製の実験台上に仰臥位で固定し、開口状態を保持した。上顎左側第一臼歯咬合面にカーバイトバーにてガイドグルーブを形成後、マイクロプラススクリュー(頭部径 1.7 mm、頭部厚 0.5 mm、全長 3.5 mm)植立し、対合する下顎左側第一臼歯根分岐部の歯根膜を観察した。なお、対照として無処置のマウスの同部位を用いた。下顎左側第一臼歯近心から遠心方向に前頭断切片を作製し、実験開始後 4 日目、7 日目、14 日目の根分岐部歯根膜における細胞動態の経時的変化を、病理組織学的ならびに免疫組織化学的検討を行い、細胞核占有率および陽性細胞率を Photoshop によって画像解析した。実験期間中のマウスの体調は良好で、体重に大きな変動はなく全身的に良好に経過した。

【結果】病理組織学的検討から、対照群と比較し実験 4 日群は、歯根膜の充血傾向、および円形の細胞核を有する細胞の密度が上昇していた。実験 7 日群は実験 4 日群と比較して、歯根膜の細胞密度は低下していたが、歯根膜中央部における多核巨細胞の出現とセメント質および歯槽骨表面には蚕食性の吸収窩が形成されていた。実験 14 日群には、多核巨細胞における骨吸収窩は拡大していた。根分岐部歯根膜における細胞核占有率は、対照群と比較し実験 4 日群、7 日群、14 日群共に増加した。とくに実験 4 日群は有意に増加していた(Scheffe 検定、$p < 0.05$)。実験 7 日群および実験 14 日群は対照群との有意差を認められなかった。免疫組織化学的検討から、Ki67 陽性細胞率は、実験 4 日群(Av±SD:17.2±4.1)に対照群(Av±SD:4.4±2.2)と比較して有意な増加がみられ(Tukey 検定、$P<0.05$)、実験 4 日群と比較して低減傾向にあるものの、実験 7 日群(Av±SD:14.7±2.2)でも有意な値を示し(Tukey 検定、$P<0.05$)、実験 14 日群(Av±SD:9.0±3.7)では、有意差はない(Tukey 検定、$P>0.05$)ものの対照群と比較して増加していた。GFP 細胞陽性率は、対照群(Av±SD:8.6±1.8)と比較して、実験 7 日群(Av±SD:19.7±6.8)で約 2.3 倍の値を示した。実験 4 日群(Av±SD:7.7±1.6)、14 日群(Av±SD:7.6±2.7)では、対照群と同程度であった。

【考察】咬合性外傷歯の共通する臨床所見は、歯の振動と動揺で、咬合時の振動、歯ぎしり時の歯の動揺は、歯周組織に過大な力が負荷されたことを意味する。マウスの下顎運動サイクルは、比較的単純であり、過重咬合時に加わる咬合圧を歯軸方向に負荷することにより、実験系を単純化することができ、染色方法が多岐にわたり分析しやすい。本研究では、飼育が比較的容易なマウスを用いて、再現性を有する実験系を確立した。過高状態を均一に設定にするために、頭部高径に規格統一性のあるマイクロプラススク

リューをマウスの上顎第一臼歯咬合面に植立した。さらに実験期間中の脱離はスクリューによって認められなかった。

Ki67 細胞陽性率は、実験 4 日群では、対照群と比較し約 2 倍の値であった。Ki67 は、細胞周期関連核タンパク質で、増殖中の細胞において発現が認められるが、増殖を休止している細胞には認められないため、増殖細胞を検出する際に使用される。このことから実験 4 日群では、外傷を受けた歯の根分岐部歯根膜に対して、活動性の細胞が多数存在することを意味しており、恒常性維持に関与すると推察できる。

さらに GFP 陽性反応の所見から、GFP 陽性細胞は、実験 7 日で、コントロールと比較して約 2 倍の増加を示したが、実験 14 日では、コントロール群とほぼ同値を示した。GFP 骨髄移植マウスは、移植した骨髄由来細胞がどのような細胞に分化しても、GFP タンパクを有しているため、生体内追跡が可能である。骨髄移植後のマウスの歯周組織に移動する細胞の細胞種を同定する研究で GFP 陽性細胞が多数移動していることが報告されており、その細胞も破骨細胞とマクロファージと同定されている。今回の実験において、GFP 骨髄移植マウスによる咬合性外傷の根分岐部における歯根膜では、実験 7 日群で、骨髄由来細胞が増加していることがみられた。これは、歯根膜に負荷される継続的な過重咬合により、受傷部位による細胞群だけでは、組織障害へ対応できずに、骨髄由来細胞の積極的な動員を必要とする現象を誘起すると考えられる。対照群の歯根膜においても GFP 陽性細胞の存在が認められることから、外傷性咬合によるこれらの骨髄由来細胞の増殖も考えられる。

以上から、外傷性咬合により惹起される咬合性外傷の根分岐部歯根膜における受傷部位では、細胞動態の亢進を伴う経時的な歯根膜の改造現象が実験 4 日から誘起されることが示唆された。さらに過重咬合状態が継続する実験 7 日をピークに歯根膜において骨髄由来細胞の増加が認められ、その後の実験 14 日では対照群とほぼ同様の組織学的所見が認められた。したがって、咬合性外傷を発症する歯根膜は、その部位の細胞お骨髄由来細胞の動員により、組織恒常性の維持が図られることが示唆された。

Cytological Kinetics of Periodontal Ligament in an Experimental Occlusal Trauma Model

Tatsuo Takaya[1], Hiroaki Mimura[1], Saeka Matsuda[2], Keisuke Nakano[2], Hidetsugu Tsujigiwa[3], Mihoko Tomida[4], Norimasa Okafuji[2], Takeo Fujii[1], and Toshiyuki Kawakami[2]

1 Department of Oral Health Promotion, Matsumoto Dental University Graduate School of Oral Medicine, Shiojiri, Japan
2 Department of Hard Tissue Research, Matsumoto Dental University Graduate School of Oral Medicine, Shiojiri, Japan
3 Department of Life Science, Faculty of Science, Okayama University of Science, Okayama, Japan
4 Department of Oral and Maxillofacial Biology, Matsumoto Dental University School of Dentistry, Shiojiri, Japan

Abstract: Using a model of experimental occlusal trauma in mice, we investigated cytological kinetics of periodontal ligament by means of histopathological, immunohistochemical, and photographical analysis methods. At furcation areas of molar teeth in the experimental group on day 4, the increase of cells in periodontal ligament was seen. The cells with a round-shaped nucleus were deeply stained in the hematoxylin-eosin. Ki67 positive nuclei showed a prominent increase in the experimental groups on days 4 and 7. Green Fluorescent Protein (GFP) positivity also revealed cell movement, but this action was slightly slow compared to Ki67 staining specimens. It was indicated that the homeostasis is mechanism of periodontal ligament receiving the excessive occlusal loading was promoted by osteoclasts and macrophages from bone-marrow-derived cells migrated to the periodontal ligament. The remodeling of periodontal ligament with the acceleration of cell growth was evoked from the experiment for the group on day 4 and after day 7. The findings suggested that periodontal ligament at furcation areas of molar teeth in occlusal trauma model was recovered by using both of the cells in situ and the bone-marrow-derived cells.

Key words: Occlusal trauma, Periodontal tissue, Green fluorescent protein (GFP), Ki67, Mouse

Introduction

Occlusal trauma is defined as an injury resulting in tissue changes within the attachment apparatus as a result of occlusal forces. It has been proved in many studies that occlusal trauma can cause a variety of destructive biological effects on periodontal tissues[1-4]. It has been suggested that occlusal trauma causes various destructive effects on the periodontal tissue, and two theories have been proposed relating to occlusal trauma: one is the "co-destructive factor theory" by Glickman[5], which suggests inflammatory changes induced by infection with periodontal pathogens and occlusal trauma caused by excessive occlusal loading both greatly contribute to the progression of periodontal diseases, especially those characterized by a large amount of alveolar bone resorption; and the other is the theory proposed by Waerhaug[6], which denies occlusal trauma as a co-factor in the loss of connective tissue attachment and vertical alveolar bone resorption. More recently, studies on animal models have examined research concerning occlusal trauma using monkeys[7]. The experiment combined different levels of inflammation and different types of trauma. Specifically, the authors ligated the molars of monkeys using a cotton thread to induce severe inflammation, and then the monkeys were divided into two groups: one received traumatic force and the other nothing. The resulting histopathological reports indicated the more severe the periodontal tissue inflammation, the greater the tissue destruction that resulted from the traumatic occlusion, and that more severe tissue destruction occurred when traumatic force was applied in two directions, rather than one[7]. Experimental mechanical stress causes changes in periodontal tissues. This has been reported by previous study[8] from an orthodontic point of view. According to the results, the changes in the pressured side of periodontal tissues were severe. However, if the cytotoxic stress is in short term, the mechanism for repairing the periodontal ligaments could be observed[8]. There are still many unknowns because experimental validations have been performed mostly from a histopathological standpoint, and experimental validation of the cellular kinetics of periodontal tissues in an *in vivo* experimental system has not made progress[7, 9-13]. These research reports failed to performed sufficient validation on the cytological kinetics of the periodontal tissues in the case of occlusal trauma. In the view of establishing an animal experimental system that is highly versatile and repeatable, we built an experimental system in which overload is added to the molar region of mice, and we reexamined the periodontal tissues from the

viewpoint of cytological kinetics[14]. We then performed histopathological and also immunohistochemical examinations.

Materials and Methods

1) Experimental animals

Eleven 7-week-old ddY male mice (weighing 35 ± 2 g) (Japan SLC Inc., Hamamatsu, Japan) and eight 7-week-old bone marrow transplanted female C57BL/6 genealogy mice (weighing 35±2 g) from GFP transgenic mice (GFP mice), for a total of nineteen mice, were used in this study (Table.1).

GFP mice were 7-week-old female C57BL/6 recipient mice (Charles River) and 7-week-old female GFP transgenic mice (C57BL/6-Tg (CAG-EGFP)) (Shimizu Lab. Supplies Co., Ltd., Kyoto, Japan).

To prepare for bone marrow transplantation, GFP transgenic mice were sacrificed under general anesthesia by isoflurane inhalation, and immediately we extracted the femur and removed the soft tissue, and harvested donor bone marrow cells suspended in RPMI 1640 medium plate with anti-biotic, displacement HBBS immediately after 7-week-old female C57BL/6 recipient mice had undergone 10 Gy of lethal whole-body-irradiation split and 1×10^7 bone marrow cells were injected into the tail vein of the recipients[15-19].

The recipient GFP mice were used 5 weeks after transplantation. The mice were kept in an air-conditioned room with controlled temperature at 24 ± 1 °C. The mice were housed in a breeding room with a 12-hour cycle of day and night and controlled in paper-lined plastic cages (Paper Clean: Peparlet Co., Ltd., Hamamatsu, Japan). The mice were freely fed with solid food (Picolab Rodent Diet 20; Japan SLC Inc., Hamamatsu, Japan) and water. The physical condition of the mice was good and there were few fluctuations in their weight during the examination.

2) Experimental methods

Each mouse was placed on a hand-made experiment table in a dorsal position under general anesthesia by intraperitoneal injection of Somnopentyl® 40 mg/kg (Pentobarbital sodium, Kyoritsu Seiyaku Corp., Tokyo, Japan). Using a #1/4 jet carbide bar (#432296 1/4, Shofu Inc., Kyoto, Japan) and a straight hand-piece drill, we created a guiding hole in the occlusal surface of upper left first molar. A micro-plus-screwpin (head part: 1.7 mm in diameter and 0.5 mm thickness, Ohsato, Saitama, Japan) was screwed into the guiding hole and fixed to the tooth. The occlusal surface of the upper left first molar was raised

by the 0.5 mm thickness of the head of the micro-plus-screwpin (Fig.1).

R_mCT was used to confirm the occlusal contact between upper left first molar and lower left first molar[14]. At 4, 7, and 14 days after increasing occlusal height, the mice were sacrificed by an overdose of pentobarbital sodium. Five mice served as a control group. A total five of ddY and C57BL/6 genealogy mice served as each experimental group on days 4, 7, and 14 (Table.1). Specimens containing the furcation area of the lower-left-first molar were fixed in 10 % neutral buffered formalin solution, demineralized in 10 % EDTA, dehydrated in increasing series of alcohol in a routine manner and embedded in paraffin. Bucco-lingual serial sections of 4 μm thickness were prepared and stained in hematoxylin-eosin. We used left first molar periodontal ligament of normal mice in the control group.

The ethics committee on laboratory animals at Matsumoto Dental University approved the examination (Number #233-13)

3) Histopathological examination

Histopathological changes of the periodontal tissues at the furcation area of the lower left first molar and its surrounding periodontal tissues were observed under a light microscope. We noted the small change form of cell nuclei and performed digital image analysis using Adobe® Photoshop CC 2014 (Adobe Systems Software Ireland Ltd., CA, U.S.A) to confirm the number of cells of the periodontal ligament at the furcation area of the lower left first molar.

4) Immunohistochemical examination

For immunohistochemical staining, the slides were deparaffinized in xylene followed by antigen retrieval in 10 mM citric acid buffer solution, pH 6.0 at 121 °C for 5min. After deparaffinization, it was blocked in 3% hydrogen peroxide for 10 min in order to devitalize endogenous peroxidase.

About Ki67 staining, the primary antibody was monoclonal Rat Anti-Mouse Ki-67 Antigen, Clone TEC-3 Code No. M7249（DakoCytomation, Denmark） with a dilution of 1:100, reaction overnight at 4°C and the secondary antibody was monoclonal mouse antibody simple stain mouse MAX-PO(M) (Nichirei Co. Ltd., Tokyo, Japan). After washing by PBS and DAB staining, specimens were counterstained with hematoxylin. For negative control, PBS was used instead of primary antibody. We counted the number of dyed cells in the periodontal ligament of the optional area.

About GFP staining, the primary antibody was Anti-GFP antibody-ChlP Grade ab290 (abcam®, Cambridge, UK) with 1:5000, overnight at 4°C and the secondary antibody was rabbit polyclonal antibody simple stain mouse MAX-PO(R) (Nichirei, Tokyo, Japan). After washing by PBS and DAB staining development, specimens were counterstained with hematoxylin. For negative control, PBS was used instead of the primary antibody.

5) Digital Image Analyze and Statistical Analyze Methods

For semi-quantitative evaluation of histopathological at immunohistochemical staining, the following procedure was performed. First, the histopathological-photographic images with same magnification from the related examination were prepared and one pixel density was counted for each image. Then, typical staining (hematoxylin; IHC-DAB) part was defined as position area. The pixel number percentage of the positive area was compared with the total pixel number percentage of the same area, and the ratio was obtained. The statistical analysis was Tukey Test or Scheffe Test by using Excel or SPSS[20]. The analyzed area of the cement-enamel junction (CEJ) in bucco-lingual position of the furcation area was drawn with a straight line, and a perpendicular line was drawn from CEJ to the alveolus bone. Cell nuclei were picked out and we calculated the pixel share of area of the cell nuclei part (Fig.2). Further, we excluded a gap in a blood vessel cavity in this analyzed part.

Result

In this experiment, the histopathological difference in the ddY mouse and the GFP mouse was not recognized. Throughout the experimental period, no inflammatory reaction was observed at the epithelium around the respective teeth.

1) Histopathological examination

In the control group, the periodontal ligament maintained a constant width, and main fibers ran across the cementum and the alveolar bone in an orderly manner. The fibroblasts in the periodontal ligament appeared spindle shaped among the collage bundles. Cell nuclei existed densely relatively, periodontal ligament fiber had the part of a minute capillary, and an erythrocyte was filled with in the blood vessel cavity. Furthermore, a cellular cementum clearly existed (Fig.3-A).

In the experimental group on day 4, the periodontal ligament was somewhat tightly compressed and the capillary hyperemia spindle evident. The spindle cell was increased in number. The cell was deeply stained in hematoxylin-eosin and had a round shaped nucleus. Multinucleate giant cells generally appeared adjacent to the alveolus bone surface. These cells were closely related to absorb the undermining bone tissue, making some lacunae (Fig.3-B).

In the experimental group on day 7, the cells with a round nucleus was decreased compared with the experimental group on day 4. Hyperemia in the blood vessels was observed in the periodontal ligament tissue. Howship's lacunae formation was appeared at the surface of the alveolus bone and the cellar cementum, respectively. Hyaline degeneration enlargement was also observed in the specimen from day 4. Furthermore, a cellar cementum breakdown was evident at the furcation of the periodontal ligament surface (Fig.3-C).

In the experimental group on day 14, the resorption area on a cellar cementum and the alveolar bone surface accompanied with multinucleated giant cells were expanding rapidly. The cells with a round nucleus decreased. The nuclei and the cytoplasm, both of which indicate the shape of the spindle, were seen again. The width of periodontal ligament became wide (Fig.3-D).

In the cytological kinetics method, we analyzed the nuclei share of pixels to compare the all pixels of the area, using the photographic nucleic analysis (hemotoxic-deeply-stained-portion) defined in the related periodontal tissue, both in the experiment and control specimens. In the all experimental groups, the results were the following: the pixel share of related area of the control group (6.7 ± 1.6%), experimental group on day 4 (11.3 ± 1.2%), day 7 (9.3 ± 2.1%) and day 14(9.3 ± 1.6%). Especially, experimental group on day 4 showed a significant increase (Scheffe Test, $p<0.05$). Compared with experimental group on day 4, experimental group on days 7 and 14 decreased but mostly same degree share and they were not significant to compare with the control group (Scheffe Test, $p<0.05$) (Fig.4).

2) Immunohistochemical examination observations

Ki67 staining, in control group, Ki67-positive-cells were hardly seen, with a small round-shape in the periodontal ligament. In the experimental group on day 4, Ki67-positive-cells increased in number and had a round shape stained in Ki67 deeply. In the experimental group on day 7, Ki67-positive-cells

decreased in number but showed a spindle shape indicated by deep staining. In the experimental group on day 14, the number of Ki67 positive cells became fewer and were not different from that of the control group. According to the digital image analysis method, we decided on the range (118×54mm) and counted the number of cells in the range and then divided the number of Ki67-positive cell by the number of overall cells. The number of Ki67 positive cells in the periodontal ligament at an experimental group on day 4 (17.2 ± 4.1%) had increased significantly compared with the control group (4.4 ± 2.2% (Tukey Test, $p<0.05$). At the experimental day 4 and 7, the Ki67-positive-cell ratio had showed a significant increase compared to that of control group (14.7 ± 2.2%) (Tukey Test, $p<0.05$). In the experimental group on day 14 (9.0 ± 3.7%), there was not significant but it increased compared with the control group (Tukey Test, $p<0.05$) (Fig.5,6).

GFP staining, at the experimental group on day 4 and day 14, a few numbers of GFP-positive-cells, which had a round shape, were observed in the periodontal ligament of the control group. According to the digital image analyze method, in the experimental group on day 7, GFP-positive-cells increased in number and a round-shape stained by GFP deeply. At the experimental day 4, the share ratio by GFP-positive-cell in periodontal ligament GFP-positive-cell pixel share of periodontal ligament at the experimental group on day 7 were increased about 3 times higher than that of the control group (Fig. 7, 8).

Discussion

It is known that traumatic occlusion in the presence of periodontal tissues along with plaque-induced inflammation may have an important contributory role in the progression of periodontal disease[6]. The periodontal ligament and histopathology-like consideration is mostly experimental of rats, macaque monkeys and beagle dogs as seen in animal experiments performed so far[11-13, 21-23]. However, the report did not find a focus point at cytological kinetics of periodontal ligament during the excessive occlusal loading. Thus, we focused the cytological kinetics in the periodontal tissues by excessive occlusal loading.

In several studies, an occlusal trauma experimental model has been carried out using various animals. Experimental *in vivo* models had used wrought crowns, casting inlays, or orthodontic square wires attached to the maxillary posterior teeth with mathylmethacrylate resin, which lead to the bite-up and

occlusal trauma for the related teeth[11-13, 21-24].

In this study, using an experimental model of occlusal trauma in mice, we analyzed histopathological and immunohistochemical changes of cytological kinetics in periodontal ligament of the lower left first molar[14].

The common clinical evidence of occlusal trauma teeth is vibration and destabilization of the teeth, and the vibration at the time of occlusion and the destabilization of teeth at the time of grinding mean that periodontal tissue is burdened with an excessive force. Wentz[25] explained that "jiggling force" was an external force shaking a tooth which was generated because the tooth crown was burdened by a force from one direction in a certain instant, and subsequently from the opposite direction in next instant, and these movements were repeated. Therefore, in order to avoid the complicated definition of "jiggling force", we used the experimental mice model to observe changes of the periodontal tissue at the time of the excessive occlusal loading. A mandibular movement cycle in mice has little grinding. The experimental model could be simplified by bite pressure under occlusal loading in the tooth axis direction[26]. Mice experiment also has other advantages such as easy breeding and many types of staining methods. It is easily to set the high state of the bite position uniformly by implanting a micro-plus-screwpin into the tooth crown. During the experiment period, the possibility of a detachment of a micro-plus-screw pin was reduced using tightening torque. Thus, use of an experimental model on an extremely small animal, in which the intermittent loading is strong enough, is indispensable and allows investigations of periodontal tissue reaction on mechanical loading in detail.

Histopathologicaly, the hyperemia in the blood vessels was observed in the periodontal ligament at the furcation area of the experimental group on day 4. A hyaline degeneration was seen in in the experimental group on day 7. This phenomenon was caused by excessive occlusal loading during the experimental period.

The previous study had investigated the bonding system of a fixed steel wire onto the occlusal surface of upper first molar without the inflammatory condition in periodontal tissues. The histopathological changes were observed, width of the periodontal ligament was measured correctly during excessive occlusal loading up to 14 days[24]. Although there was difference in experimental periods or animals, that of the previous studies, the histopathological changes were similar to our results. The experimental mice model was useful for analysis of the occlusal trauma pathogenesis. The cell

density of the periodontal ligament increased significantly in the experimental group on day 4. This phenomenon suggests that the tissue received by excessive occlusal loading has a recovery potential. The duration of an excessive occlusal loading caused a hyaline degeneration and some other damage in periodontal ligament. These observations suggested the possibility of cell extinction in the injured periodontal ligament. In the experimental group on day 7, the pixel share of nuclei of periodontal ligament cells showed a slight decrease. In experimental group on day 14, there were no significant differences between the experimental and control group. Thus after receiving occlusal trauma, periodontal ligament showed the tissue adaptation against excessive occlusal loading.

The Ki67-positive cells in periodontal ligament of the experimental group on day 4 had a value of approximately two times than that of control group. Ki67 is a related nucleoprotein in the cell cycle. Therefore, these observations suggested that the activity of cell division existed in periodontal ligament which received an injury caused by excessive occlusal loading. In the present study, cell count was determined by counting GFP-positive-cells in the control and experimental groups. From observations of GFP-staining specimens, the share ratio of GFP-positive cells had a peak value at the experimental group on day 7. It is reported in the result of previous studies that GFP-positive-cells migrated to the periodontal tissues of the mouse after a marrow transplant and such cells identified with osteoclasts and macrophages[27]. The previous study showed that transplanted bone-marrow-derived cells migrated to the area of bone repair shown by double staining with GFP and CD34 in osteoclasts, etc[19]. The numbers of the bone marrow-derived cells migrated into the periodontal ligament at the furcation area of occlusal trauma increased with GFP mouse of the experimental group on day 7. It is suggested that the GFP-positive-cells are osteoclasts and macrophages by the previous study[19]. At the injury area caused by continuous excessive occlusal loading, not only periodontal ligament dominant cells but also bone-marrow-derived cells play an important role during the adaptation period of the tissue. These results study suggested that occlusal trauma promoted enhance of cytological kinetics of periodontal ligament in the early phase of excessive occlusal loading. The continuative remodeling with the acceleration of the cell proliferation is induced in the injury part of periodontal ligament by occlusal trauma. The findings of this study indicate that both of periodontal ligament dominant cells and bone-marrow-derived cells are mobilized by the potent

cells provided with an ability to rescue the periodontal ligament from the injury after excessive occlusal loading.

Conflict of interest

The authors have declared that no conflict of interest.

Author Contributions

T. Takaya and H. Mimura contributed to conception, mouse examination, staining of H-E and IHC, design, data acquisition, analysis interpretation, and drafted the manuscript and final version of the manuscript; S. Matsuda, contributed to analyzed R_mCT; K. Nakano, contributed to conception, staining of H-E and IHC; H. Tsujigiwa contributed to conception, GFP mouse; M. Tomida, contributed to analyzed data acquisition; N. Okafuji and T. Fujii, contributed to conception, and interpretation; T. Kawakami contributed to conception, and supervision, and he critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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	Cont.	4 day	7 day	14 day	Total
No	5 (2)	5 (2)	5 (2)	5 (2)	20 (8)

( ): GFP mice

Table 1. Experimental Periods and Number of Specimens

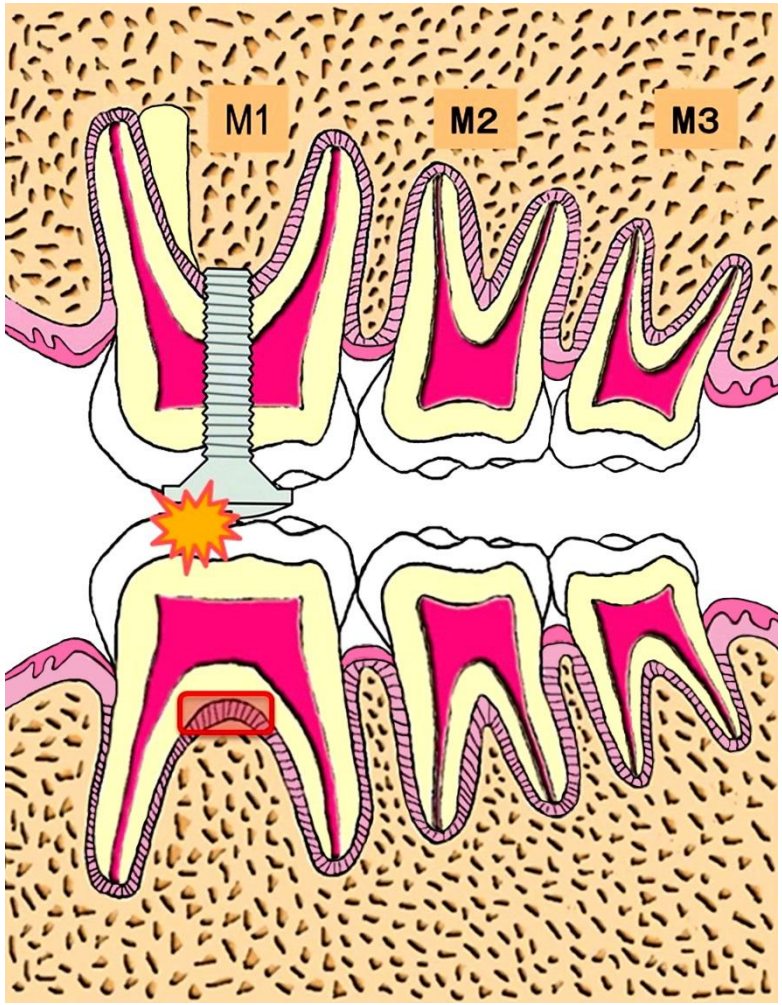

Figure 1. Experimental schema

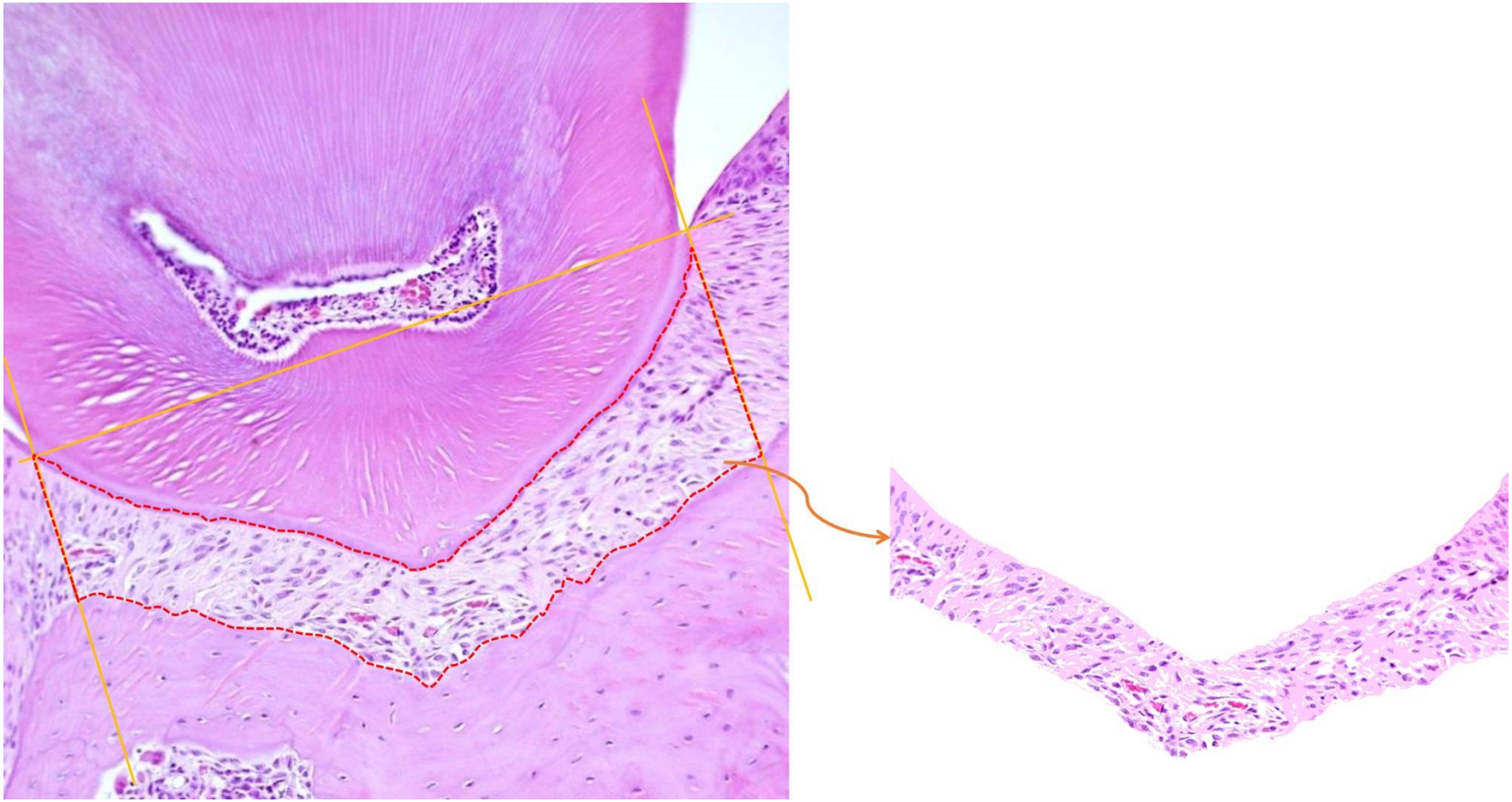

Figure 2. Histopathological photograph of the observation site

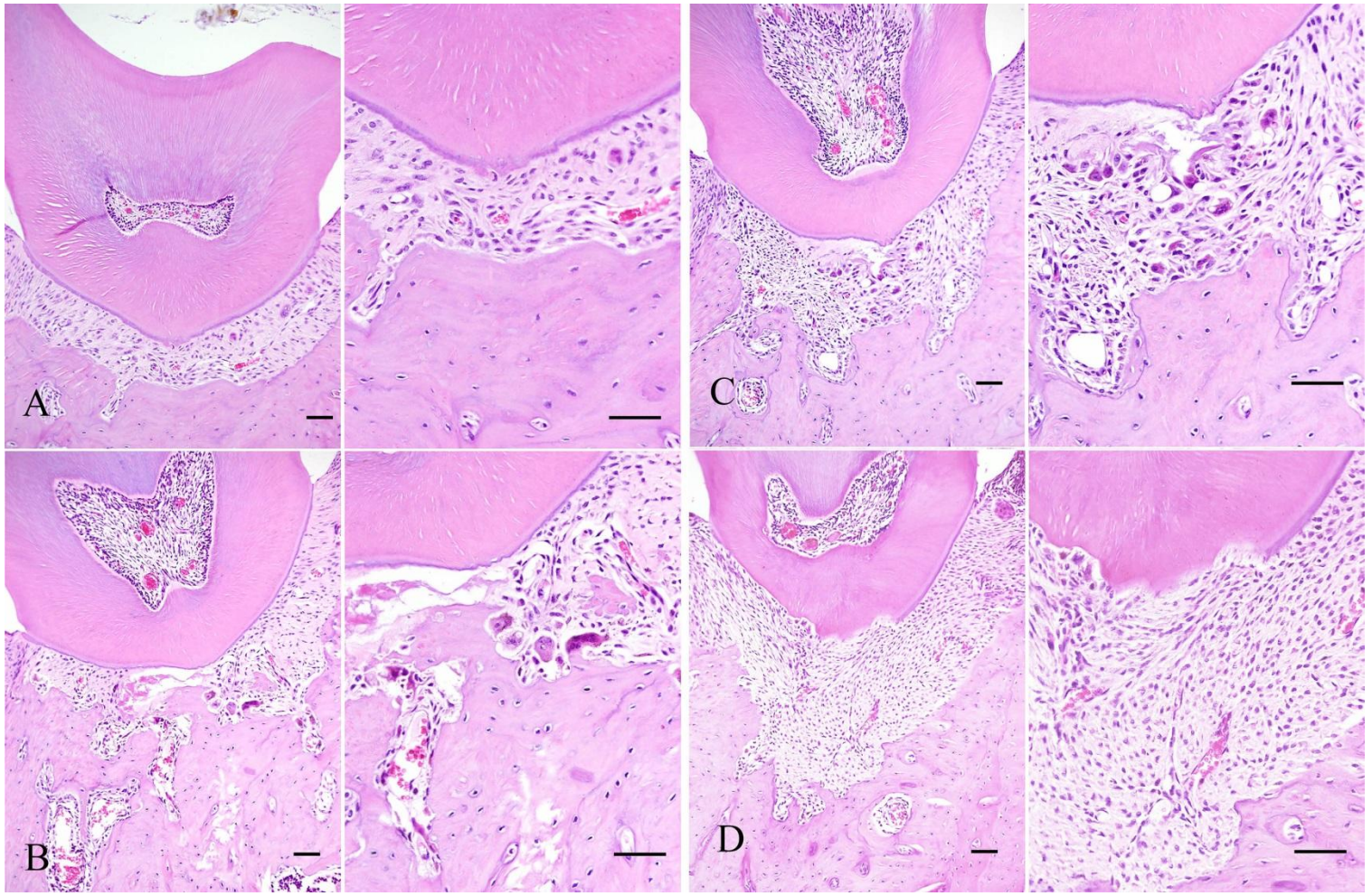

Figure 3. Histopathology of control group specimen (A), experimental 4 day group specimen (B), experimental 7 day group specimen (C) and experimental 14 day group specimen (D). Scale bar: 50 μm.

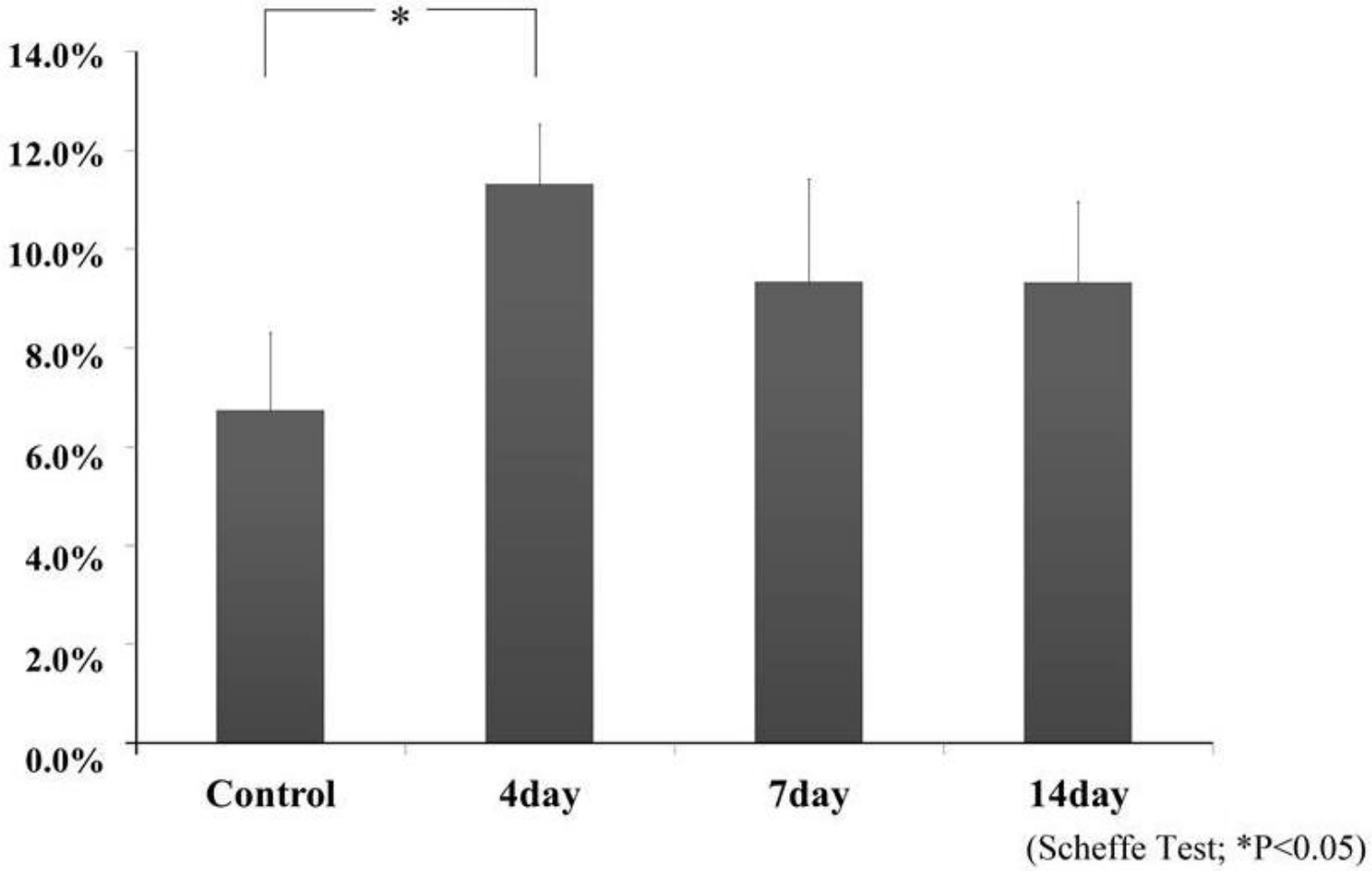

Figure 4. Hematoxylin-deeply-stained-portion sharing ratio

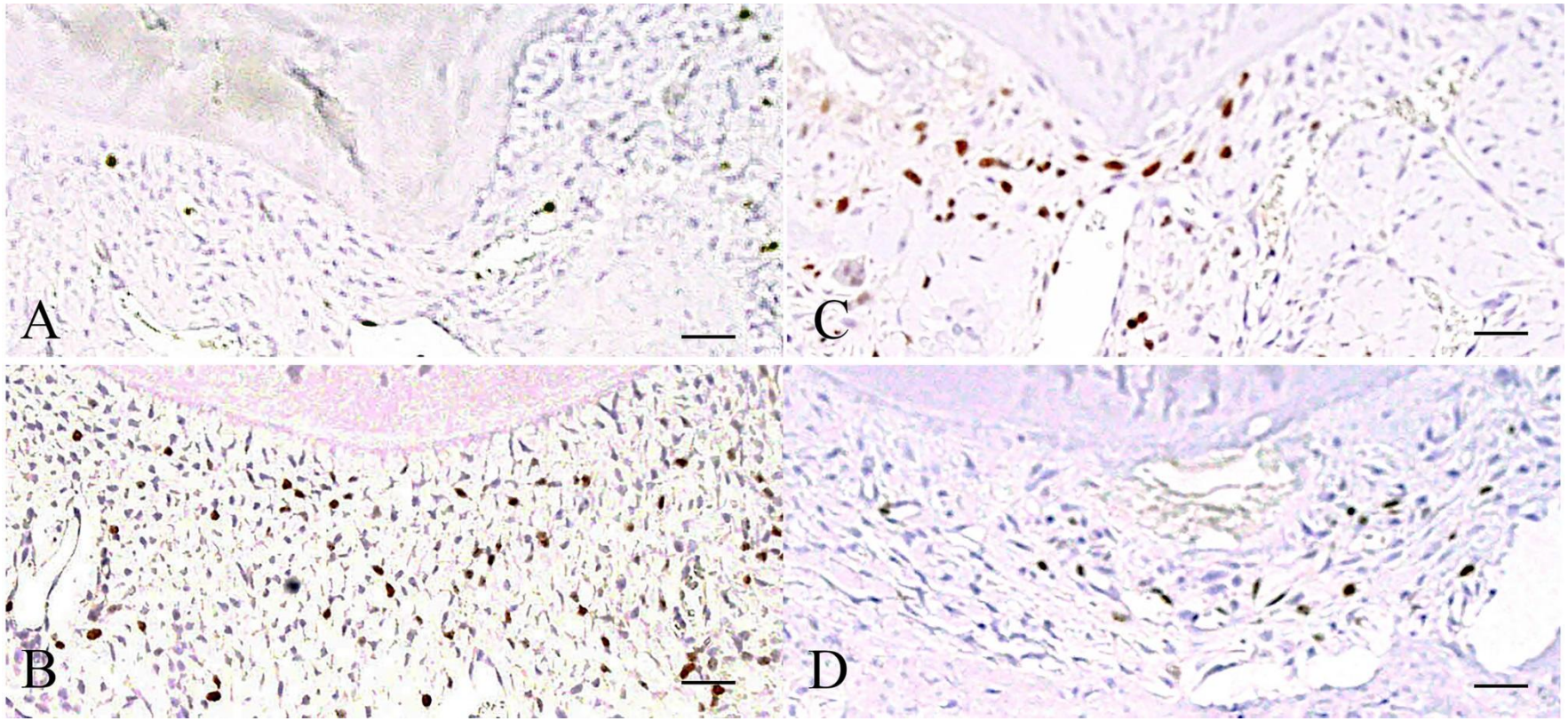

Figure 5. Ki67 IHC staining specimens. Control (A), Experimental 4 day (B), Experimental 7 day (C) and Experimental 14 day (D). Scale bar: 50μm.

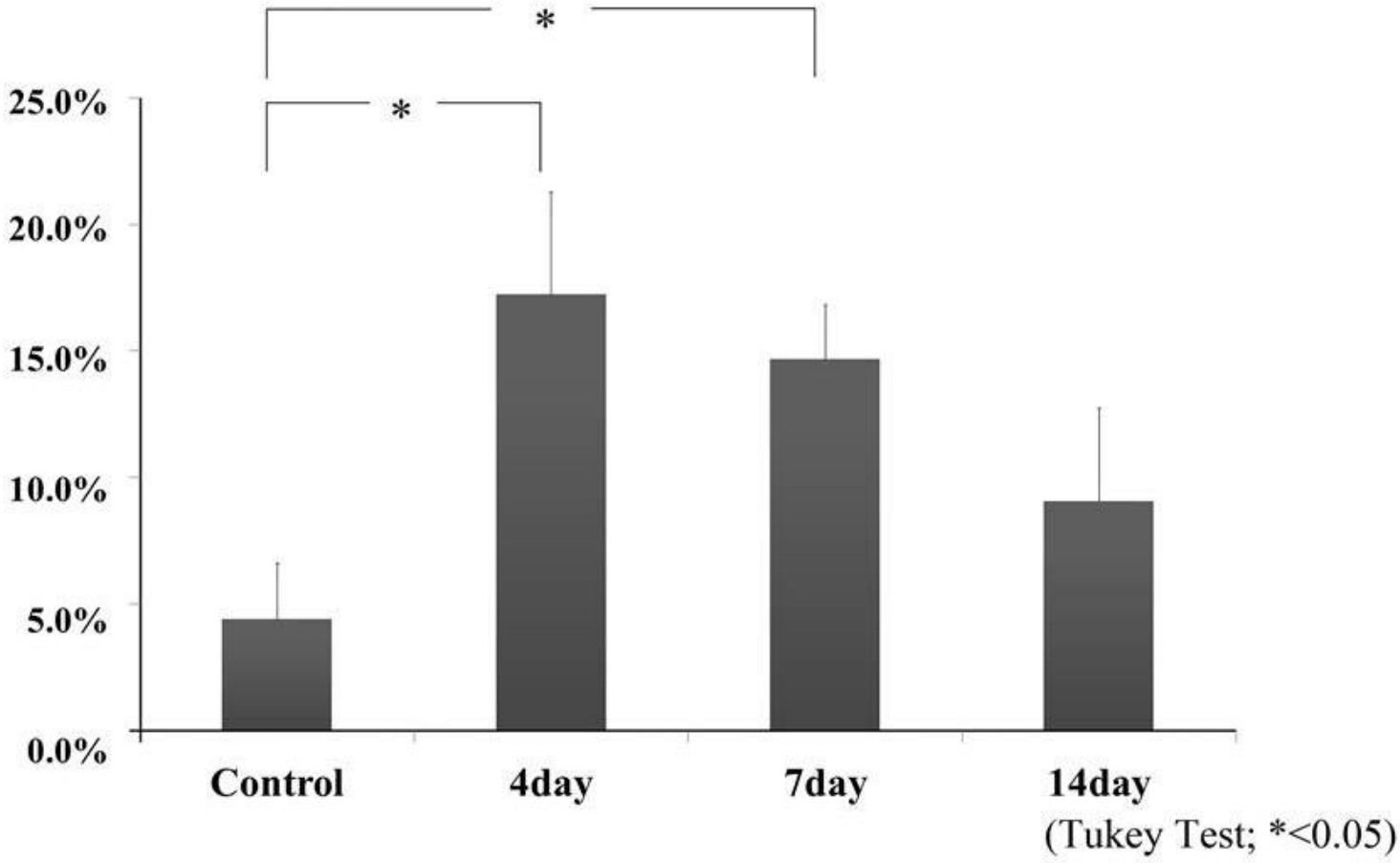

Figure 6. Ki67-positive cell ratio

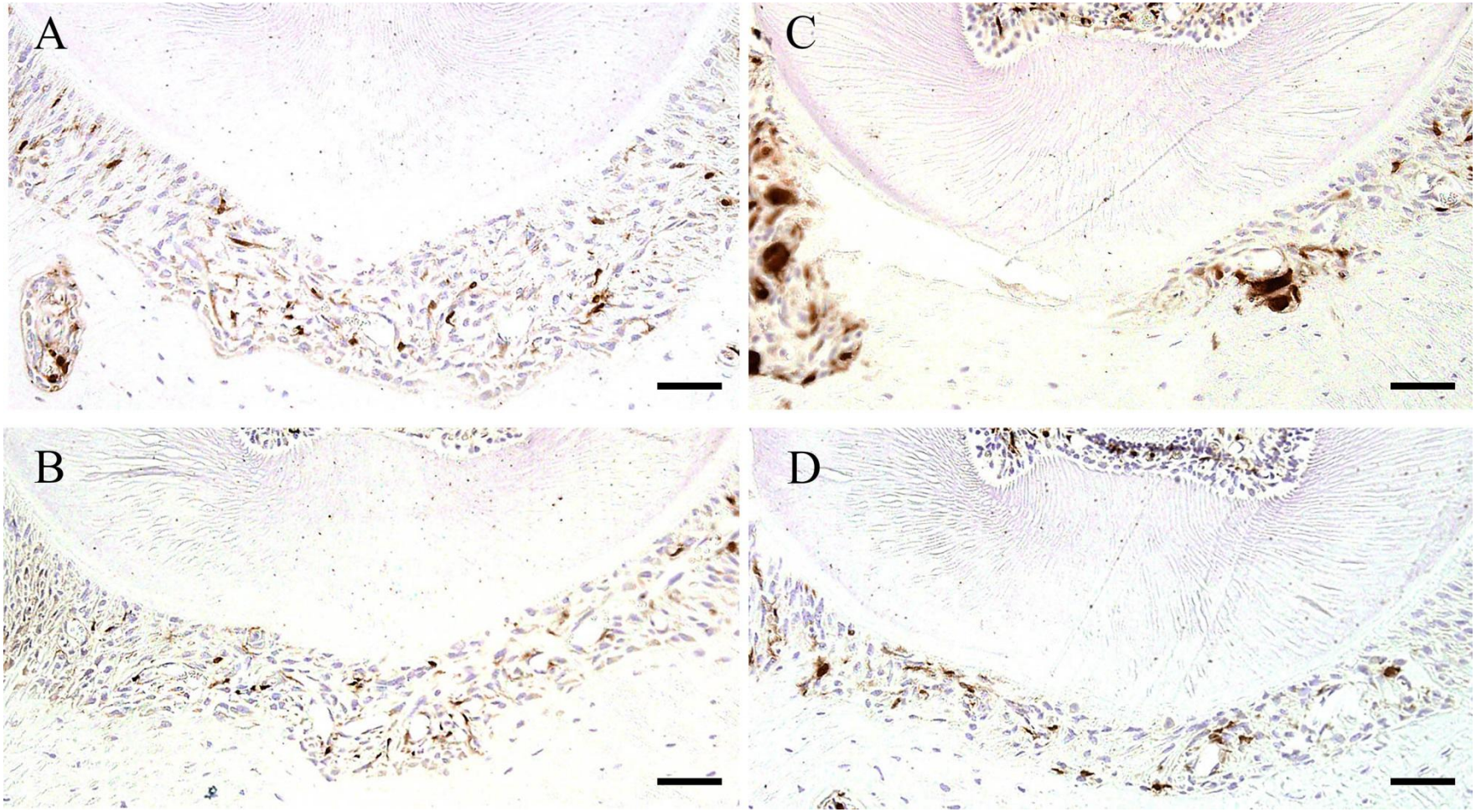

Figure 7. IHC staining of GFP. Control specimen (A), Experimental 4 day specimen (B), Experimental 7 day specimen (C) and Experimental 14 day specimen (D). Scale bar: 50 μm.

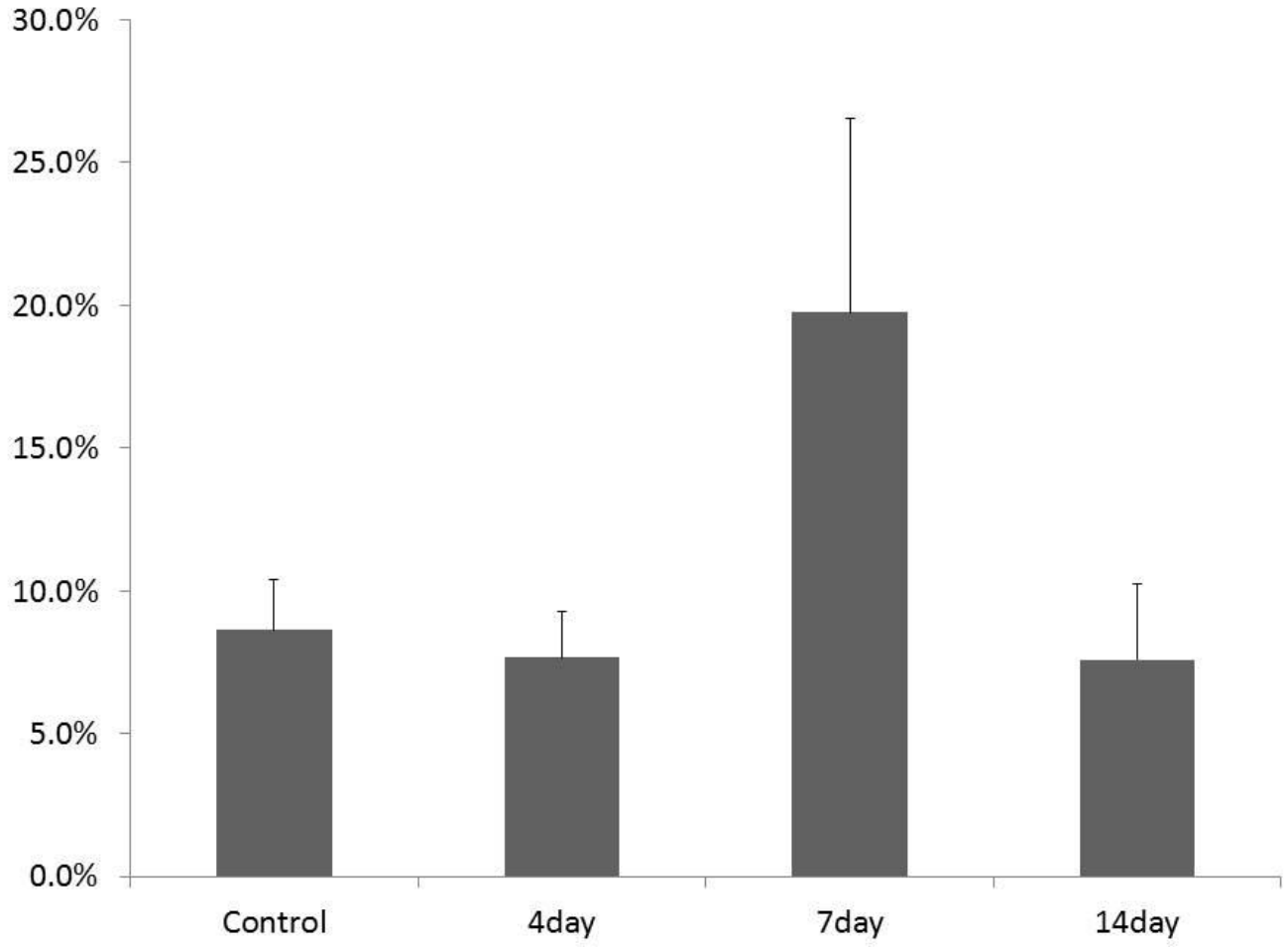

Figure 8. GFP positive cell share ratio