The Effect of Dissociated Soft Tissue on Osteogenesis: A Preliminary In Vitro Study

Sawako Kawakami, DDS[1]/Makoto Shiota, DDS, PhD[2]/Kazuhiro Kon, DDS, PhD[3]/
Masahiro Shimogishi, DDS, PhD[3]/Shohei Kasugai, DDS, PhD[4]

Purpose: *The purpose of this in vitro study was to examine the effect of dissociated soft tissue on bone marrow cell proliferation and differentiation under osteogenic conditions.* ***Materials and Methods:*** *Rat bone marrow cells were cultured to assess the stimulation of cell proliferation and differentiation. Harvested palatal mucosa was dissociated using a device (Rigenera, Human Brain Wave), and the dissociated soft tissue was cultured with rat bone marrow cells. Cell proliferation, differentiation, and mineralized nodule formation were assessed after 2 or 5 days of culturing. Bone marrow cell proliferation was assessed by quantifying the absorbance of a water-soluble tetrazolium salt using a cell proliferation assay kit. Bone marrow cell differentiation was assessed by alkaline phosphatase staining and real-time polymerase chain reaction. Mineralized nodule formation was assessed by Alizarin red staining.* ***Results:*** *At day 2, cell proliferation, osteoblast-specific gene expression, and mineralized nodule formation were significantly higher in the experimental group than in the control group. Alkaline phosphatase staining was also higher in the experimental group on day 2. Mineralized nodule formation area and osteoblast-specific gene expression were also statistically higher in the experimental group on day 5.* ***Conclusion:*** *This study demonstrates that dissociated soft tissue elevates bone marrow cell proliferation and differentiation under osteogenic conditions.* Int J Oral Maxillofac Implants 2019;34:651–657. doi: 10.11607/jomi.7021

Keywords: *bone regeneration, dissociated soft tissue solution, in vitro study, palatal mucosa*

Over the past quarter-century, implant dentistry has become a reliable common therapy standard for edentulous patients. However, implant placement is often restricted in individuals with deficient bone volume.[1–5] Therefore, autogenous bone or a bone substitute is needed as graft material for bone augmentation. These two types of graft material have advantages and disadvantages.[6–9] Autogenous bone grafts possess osteogenic ability,[10] although their volume is limited because they must be harvested from a host site. In contrast, bone substitutes eliminate the need for harvesting surgery, although they lack regenerative properties and increase the risk of immunogenic reaction.[11] Growth factors, such as bone morphogenetic protein, have also been used in clinical cases of bone augmentation.[12,13] However, the use of these proteins has been reported to cause edema, tissue damage, or fetal development complications.[14] For these reasons, an alternative, effective approach for bone augmentation is required for dental treatment.

Dissociated soft tissue has been attracting increasing attention in the field of soft tissue engineering in recent years; these applications have included the healing of wounds or ulcers, as well as plastic surgery procedures such as hair transplantation.[15] Several studies have reported that dissociated tissue, which contains enriched progenitor cells, has the ability to increase the regenerative potential.[16,17] Based on these studies, the present authors hypothesized that dissociated soft tissue includes topical growth factors, which

[1]Postgraduate Student, Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo, Japan.
[2]Associate Professor, Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo, Japan.
[3]Clinical Fellow, Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo, Japan.
[4]Professor, Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo, Japan.

Correspondence to: Dr Sawako Kawakami, Department of Oral Implantology and Regenerative Dental Medicine, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. Fax: + 81-3-5803-5774. Email: kawairm@tmd.ac.jp

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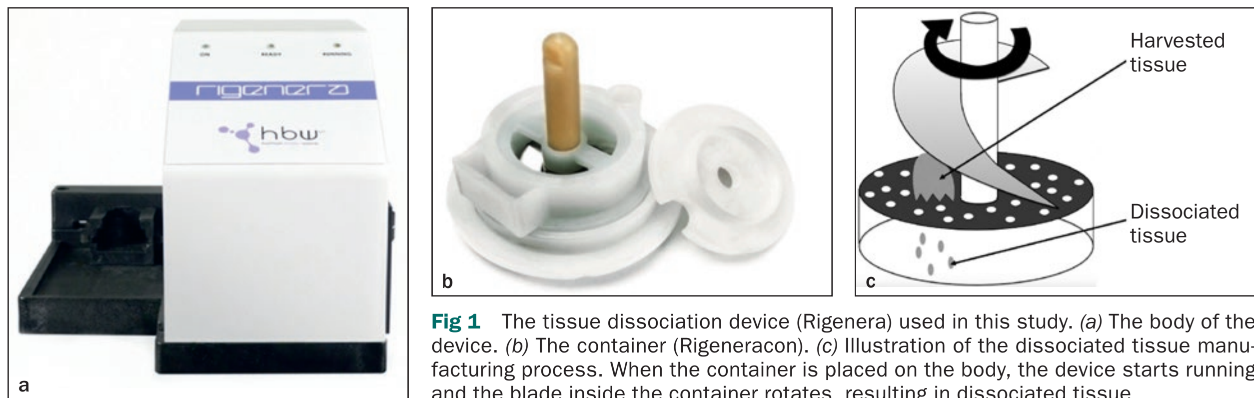

Fig 1 The tissue dissociation device (Rigenera) used in this study. *(a)* The body of the device. *(b)* The container (Rigeneracon). *(c)* Illustration of the dissociated tissue manufacturing process. When the container is placed on the body, the device starts running and the blade inside the container rotates, resulting in dissociated tissue.

enhance osteogenesis and have a beneficial effect on bone augmentation. The aim of this study was to evaluate the effect of dissociated soft tissue on bone marrow cell proliferation and differentiation under osteogenic conditions.

MATERIALS AND METHODS

All animal experiment protocols were approved by the Animal Welfare Committee of Tokyo Dental and Medical University (Approval number: 2017–104C).

Isolation and Culturing of Bone Marrow Cells

Six-week-old male Slc:Wistar/ST rats (Sankyo Lab) were used in this study. Following euthanasia, bone marrow cells were isolated from the rat femurs. The released bone marrow cells were seeded in 75-cm^2 flasks with 10 mL culture medium comprising alpha-minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Sigma-Aldrich).[18] Bone marrow cells were cultured under 95% humidity, 5% CO_2, and at 37°C. The culture medium was replaced after 2 days of culturing to eliminate nonadherent cells. Once the bone marrow cells reached 80% to 90% confluence, they were trypsinized and subcultured into fresh flasks containing the same medium.

Preparation of Dissociated Soft Tissue

Soft tissue was prepared from the palatal mucosa of another group of 6-week-old male Slc:Wistar/ST rats. Harvested palatal mucosa was dissociated using a tissue dissociation device (Rigenera; Human Brain Wave; Fig 1). Following euthanasia, approximately 3 × 4 mm of partial thickness palatal mucosa was isolated. The superficial epithelium was removed, and the remaining mucosa was cut in half (two pieces of 3 × 2 mm). Each piece of tissue was placed in a container with 1 mL medium and processed using the dissociation device for 60 seconds, in accordance with the manufacturer's protocols.

Cell Seeding

At passage 3, bone marrow cells were seeded in 24-well plates and 96-well plates for assessment. In the 24-well plates, the cells were seeded at a density of 5.0×10^4 cells/well for alkaline phosphatase staining, quantitative real-time polymerase chain reaction (qRT-PCR) analysis, and Alizarin red staining. In the 96-well plates, the cells were seeded at a density of 7.5×10^3 cells/well for the cell proliferation assay.

Following cell seeding, dissociated soft tissue was added to the experimental group culture plates, while the same amount of medium was added to the control group; 200 μL and 40 μL were added to the 24-well and 96-well plates, respectively. In both groups, the bone marrow cells were cultured in osteogenic medium comprising culture medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid.

Assessment of Cell Proliferation

Bone marrow cell proliferation was quantified with the Cell Counting Kit-8 (Dojindo). This method detects mitochondrial enzyme–mediated reduction of a water-soluble tetrazolium salt to a formazan dye. After 2 or 5 days in culture, the cells were washed and added to medium containing 10% Cell Counting Kit-8 reagents prior to incubation at 5% CO_2 and 37°C for 3 hours, in accordance with the manufacturer's protocols. The amount of formazan dye generated was measured with a microplate reader (FLUOstar OPTIMA-6, BMG Labtech). Measurements were performed at a wavelength of 450 nm.

Alkaline Phosphatase Staining

Alkaline phosphatase staining was used to qualitatively assess alkaline phosphatase activity. After 2 or 5 days in culture, the cells were washed and fixed using 10% formalin, and then rinsed with distilled water

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prior to staining. Alkaline phosphatase staining was performed using the TRAP/ALP Staining Kit (Wako), following the manufacturer's protocols. The stained cells were observed using an optical microscope (BIO ZERO BZ-8000, Keyence).

qRT-PCR Analysis

To compare the mRNA expression levels of osteoblast-specific genes, qRT-PCR analysis was performed after 2 or 5 days in culture. TaqMan probes (Applied Biosystems) were used to assess mRNA expression levels. Four target genes, Type 1 collagen (Col1; Rn01463848), alkaline phosphatase (Alp; Rn01516028), osteopontin (Opn; Rn00681031), and osteocalcin (Ocn; Rn00566386), and a reference gene, glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Rn99999916), were examined. Total RNA was isolated from each bone marrow cell sample using TRIzol Reagent (Invitrogen) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher). Complementary DNA was reverse-transcribed from total RNA using SuperScript IV (Life Technologies). qRT-PCR analysis was performed using the Step One Plus real-time PCR system (Applied Biosystems) with the TaqMan Fast Advanced Master Mix (Applied Biosystems). The samples were simultaneously denatured at 95°C for 20 seconds prior to 45 amplification cycles of denaturing and annealing at 95°C for 1 second and extension at 60°C for 20 seconds. The expression levels of the four target mRNAs (Col, Alp, Opn, and Ocn) were normalized to the expression level of Gapdh using the ΔΔCt method.

Alizarin Red Staining

Mineralized nodule formation was measured by Alizarin red staining after 2 or 5 days in culture. The cells were washed and fixed with methyl alcohol and then rinsed with distilled water. Alizarin red staining was performed using the Calcified Nodule Staining kit (Cosmo Bio), following the manufacturer's protocols. The mineralized nodules were observed using a fluorescence microscope (BIO ZERO BZ-8000) at a wavelength of 565 nm. Binary images were generated,[19] and the stained area was measured.

Statistical Analysis

All statistics were performed using the Mann-Whitney *U* test with SPSS (SPSS version 22, IBM). *P* values < .05 were considered as statistically significant.

RESULTS

Cell Proliferation Assay

The experimental group showed significantly higher proliferation than the control group on day 2 (Fig 2, *P* < .05). However, on day 5, cell proliferation remained largely unchanged between the groups.

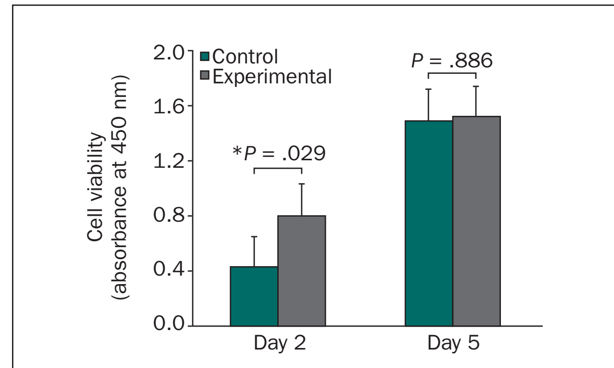

Fig 2 Measurement of cell proliferation. Data are presented as mean ± SD. Statistically significant differences were observed on day 2 (*$P < .05$; n = 4).

Alkaline Phosphatase Staining

Figure 3 shows the alkaline phosphatase staining results. The staining was observed in both groups at both time points. However, on day 2, the alkaline phosphatase staining was stronger in the experimental group than in the control group.

qRT-PCR Analysis

The expression levels of osteoblastic markers were investigated by qRT-PCR analysis. The relative expression levels of Type 1 collagen, osteopontin, and osteocalcin were significantly upregulated in the experimental group compared with the control group at both time points (Figs 4 and 5, *P* < .05). The expression level of alkaline phosphatase was higher in the experimental group than in the control group on day 2, but was lower on day 5; however, no statistically significant differences were observed.

Alizarin Red Staining

Mineralized nodules were observed in both groups after 2 or 5 days in culture by Alizarin red staining (Fig 6). Statistically significant differences were observed in the mineralized nodule area between the experimental group and control group at both time points (Fig 7, *P* < .05).

DISCUSSION

Bone augmentation provides an important means of expanding the application of implant-based treatment in edentulous patients. This in vitro study investigated the effect of dissociated soft tissue on osteogenesis by assessing bone marrow cell proliferation, differentiation, and mineralized nodule formation.

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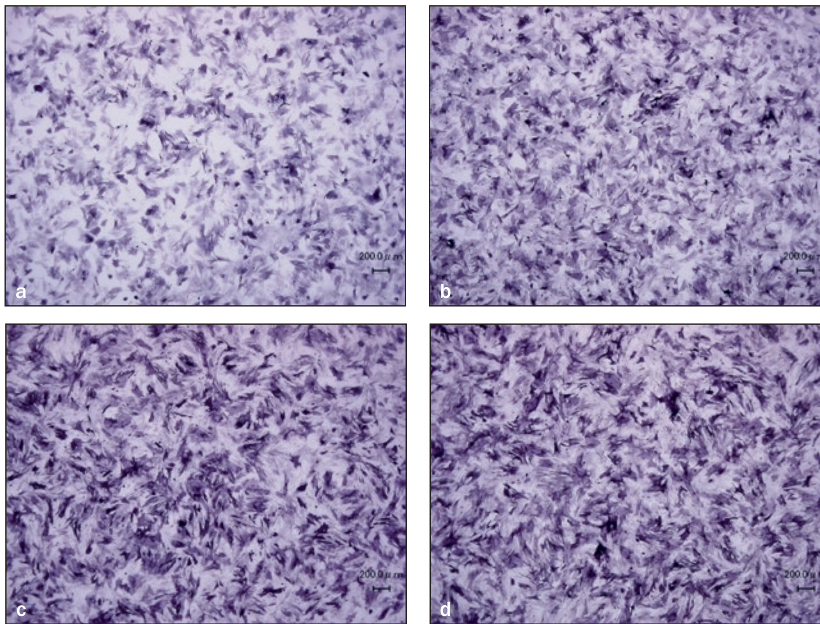

Fig 3 Alkaline phosphatase staining images in each group were observed by optical microscope (magnification ×4): *(a)* control group on day 2; *(b)* experimental group on day 2; *(c)* control group on day 5; *(d)* experimental group on day 5.

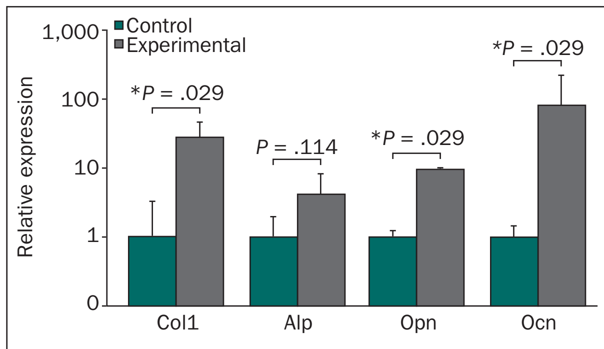

Fig 4 The expression levels of osteoblastic markers assessed by qRT-PCR on day 2. Data are presented as mean ± SD. Col1, Opn, and Ocn showed statistically significant differences (*$P < .05$; n = 4).

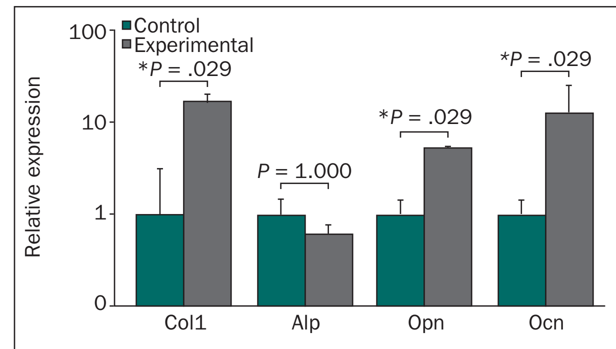

Fig 5 The expression levels of osteoblastic markers assessed by qRT-PCR on day 5. Data are presented as mean ± SD. Col1, Opn, and Ocn showed statistically significant differences (*$P < .05$; n = 4).

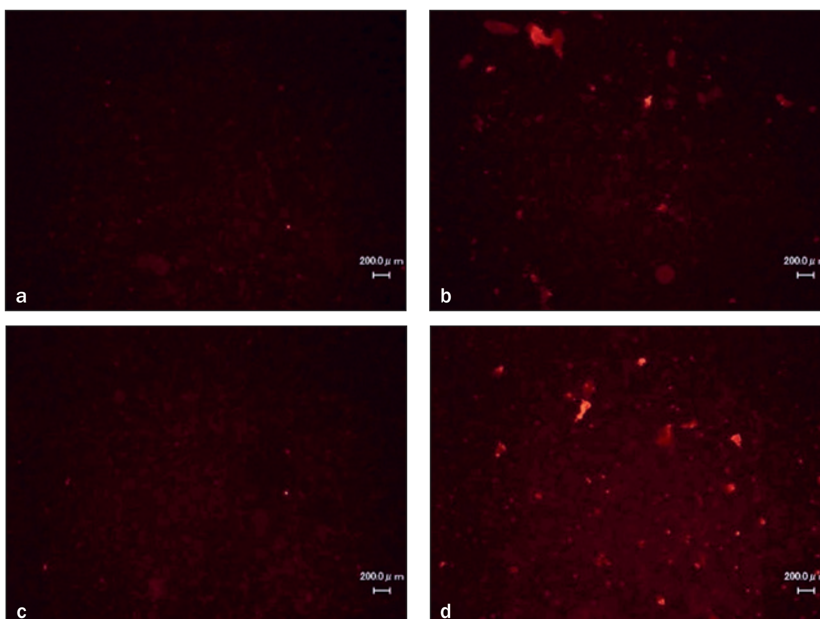

Fig 6 Alizarin red staining images in each group were observed by fluorescence microscope (magnification ×4): *(a)* control group on day 2; *(b)* experimental group on day 2; *(c)* control group on day 5; *(d)* experimental group on day 5.

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Bone marrow cell proliferation was significantly higher in the experimental group on day 2. However, no statistically significant differences were observed on day 5. These results demonstrate that the dissociated soft tissue was washed away when the osteogenic medium was changed. Thus, continued addition of dissociated soft tissue during culturing might result in higher bone marrow cell proliferation.

Alkaline phosphatase is an osteoblast marker that is expressed during the early phase of bone formation. Alkaline phosphatase staining revealed that alkaline phosphatase activity levels were elevated in the experimental group on day 2, relative to those in the control group; however, alkaline phosphatase mRNA levels remained largely unchanged between the groups on day 2. These results indicate that the high alkaline phosphatase activity may be due to high bone marrow cell proliferation.

The gene expression of bone matrix proteins varies depending on cell differentiation progress. Osteopontin is expressed in immature osteoblasts, while osteocalcin is strongly expressed in mature osteoblasts.[20] In the present study, the mRNA expression levels of both osteopontin and osteocalcin were significantly upregulated in the experimental group on days 2 and 5 compared with the control group. These results indicate that culturing bone marrow cells with dissociated soft tissue promoted their differentiation into osteoblast-like cells.

Type 1 collagen shows weak expression in mesenchymal cells and preosteoblasts, while osteoblasts express increased levels of this protein.[20] Type 1 collagen is an important extracellular matrix protein that is involved during periods of rapid proliferation or matrix synthesis[21] and during maintenance of the bone cell phenotype.[21,22] This protein induces the expression of genes such as osteopontin and osteocalcin, which accelerate mineralization of the extracellular matrix.[21,23–25] In the present study, elevated mRNA levels of Type 1 collagen were detected in the experimental group on day 2, concurrent with significantly increased bone marrow cell proliferation and mineralized nodule formation compared with the control group. These results suggest that mRNA may have been extracted from viable cells present in the dissociated soft tissue in the experimental group. d'Aquino et al have reported that micrografts, prepared using the same dissociation device (Rigenera), include rich viable cells.[16] In the present study, the dissociated soft tissue was directly added to the bone marrow cells; hence, future studies should employ a co-culture system, such as a cell culture insert, to ensure that mRNA is extracted solely from bone marrow cells.

Mineralized nodules were observed in the experimental group at both time points. Mineralization

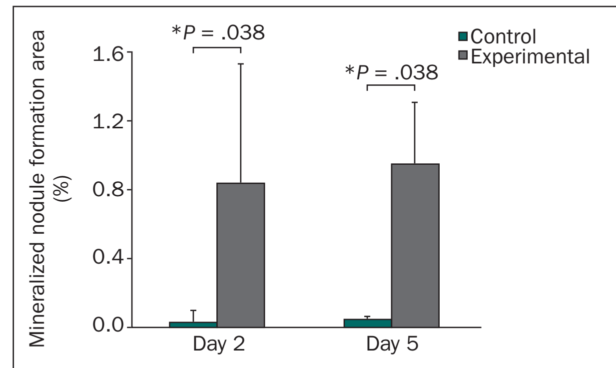

Fig 7 Alizarin red staining for measuring the area of mineralized nodules. Data are presented as mean ± SD. Statistically significant differences were observed on days 2 and 5 (*$P < .05$; n = 4).

occurs during the later stage of osteoblast differentiation. This suggests that bone marrow cell differentiation was accelerated by dissociated soft tissue at day 2. In addition, high cell proliferation and differentiation were observed in the experimental group at day 2, indicating that dissociated soft tissue induced mineralized nodule formation via a synergistic effect on cell proliferation and differentiation.

A recent study has reported a procedure for converting fibroblasts into osteoblasts by exposing them to transcription factors, such as runt-related transcription factor 2 and osterix, under particular experimental conditions.[26] Runt-related transcription factor 2 expression is elevated during the early stage of osteoblastic differentiation. Osterix expression is specific to osteolineage cells and is expressed after runt-related transcription factor 2. In the present study, the dissociated soft tissue might have contained viable cells such as fibroblasts, adipocytes, immune cells, or mesenchymal stem cells. Previous studies have reported that dissociated tissue contains active cells and progenitor cells.[16,17] In addition, transcription factors released from bone marrow cells cultured under osteogenic conditions might affect the viable cells present in the dissociated soft tissue. Further studies are required to investigate the viable cells in dissociated palatal mucosa.

A number of recent studies have reported that vascular endothelial growth factor and fibroblast growth factor 2 or 8 positively influence osteoblast differentiation and bone formation.[27–29] Noda et al showed that the expression levels of proangiogenic factors, such as vascular endothelial growth factor and fibroblast growth factors, were increased in gingival connective tissue.[30] These results might reflect the synergistic effects of viable cells and signal molecules within the dissociated soft tissue. Thus, further studies are needed to investigate the mechanisms underlying the cell proliferation and differentiation effects observed in the present study.

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This study used palatal mucosa to prepare the dissociated soft tissue, as a relatively large amount of this tissue can be harvested compared with bone. In addition, this tissue was selected because it is employed in the authors' daily clinical practice to obtain keratinized gingival tissue or treat peri-implant soft tissue recession.[31]

This study had a number of limitations: *(1)* the dissociated soft tissue was directly added to the bone marrow cells and *(2)* the dissociated soft tissue was added only once. Thus, further studies using a co-culture system, such as a cell culture insert, and repeated addition of dissociated soft tissue are needed.

The authors hypothesize that this method could be applied for bone augmentation in the future. Dissociated autologous mucosa mixed with a sustained release compound is injected or directly applied to the defect; sustained release of cells or signaling molecules will lead to early bone regeneration.

The notable aspect of this study is that dissociated palatal mucosa enhanced cell proliferation, differentiation, and mineralized nodule formation. To the best of the authors' knowledge, this is the first study to report that dissociated mucosal tissue enhances osteogenesis. To confirm this hypothesis, this preliminary study needs to add dissociated palatal mucosa to bone marrow cells. Moreover, further studies are required to investigate the factors present in dissociated mucosal tissue that enhance differentiation.

CONCLUSIONS

This study indicates that bone marrow cell proliferation, differentiation, and mineralized nodule formation are accelerated by dissociated palatal mucosa under osteogenic conditions. Further studies are required to investigate the cells or signaling molecules involved in dissociated soft tissue, which promote cell proliferation, differentiation, and mineralized nodule formation. Additionally, in vivo studies using bone defect models are required to confirm the present results.

ACKNOWLEDGMENTS

The authors declare there are no conflicts of interest related to this study.

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