In situ soft tissue regeneration using periosteal distraction: A preliminary study in the rat calvarial model

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Abstract Aim: In this study, we aimed to evaluate soft tissue generated by periosteal distraction. Background: Management of soft tissue defects represents a challenge in dentistry. Previous periosteal distraction studies documented partial fill of the distraction space with newly-generated bone and fibrous connective tissue. Material and methods: Titanium meshes were inserted in subperiosteal tunnels in the calvaria of 20 rats through coronal incision. The devices were immediately activated after insertion by elevation of one side at 1 mm/day for 3 days. Rats were then divided into two groups (n = 10). Animals were sacrificed after 2 weeks (Group 1) and after 4 weeks (Group 2). Distraction sites specimens were embedded in paraffin and analyzed histologically and histomorphometrically. Results: In both groups, new periosteum was regenerated and covered the original bone surface in the distraction site. Distraction spaces showed a predomination of hyper-vascularized connective tissue and little new bone formation near to the stable end of the device. The 4-week findings showed more organized collagen fibers with less vascularity compared to the 2-week findings. Conclusion: The periosteal distraction technique can effectively regenerate connective tissue. It may open a new modality in the guided tissue regeneration for soft tissue augmentation. © 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Soft tissue defects following teeth extraction is a well-known problem in dentistry. Soft tissue contours usually follow the architecture of the underlying resorbed bone after tooth loss, resulting in a deficiency of soft tissue (Tan et al., 2012). A horizontal pattern is the second most common pattern of a post-extraction ridge defect (Abrams et al., 1987). Hence the aes-
thetum at a fast speed in a rat calvarial model using a custom-
made titanium mesh with wide holes to expose more perios-
team. Regenerated tissue was assessed histomorphometrically
and histologically.

2. Materials and methods

2.1. Animals

Since this is the first study to report soft tissue formation using
fast periosteal distraction, no previous studies with estimated
means were available. The sample size for this preliminary
study was calculated based on the best prediction using the
mean of newly formed connective tissue thickness (1.5 m
\( \pm 0.5 \)). The sample size to ensure that a two sided test with
\( \alpha = 0.05 \) had 80% power to detect a 1.5 mm difference was
seven animals per group. We increased the sample to 10 ani-
mals to avoid a sample attrition effect.

A total of 20 male Wistar rats were included in this study
(\textit{Rattus norvegicus albinus}). Average weight of the animals
was 300–350 g. Before the experiment, the animals were
allowed to adapt in a controlled environment with a room tem-
perature of 22 °C, 12 h dark/light cycle, and access to water
and food ad libitum. The animals were divided into two
groups, each containing 10 rats. The animal experiments in
this study were performed in accordance with the guidelines
of the Helsinki Declaration and the study was approved by
the ethical committee of Tokyo Medical and Dental Univer-
sity, Tokyo, Japan (IRB0130280).

2.2. Distraction device

A custom-made titanium mesh (16 × 8 × 0.3 mm) was pre-
wared with two holes (4 mm diameter) created in each mesh.
A titanium distraction screw (5 × 1.5 mm) was used to distract
the device gradually by advancing in a serrated hole at the
movable end of the mesh (Fig. 1A).

2.3. Experimental design

The animals received a titanium periosteal distraction device
(TPDD). The elevating screw was advanced in the screw hole
of (TPDD). Activation commenced immediately after insertion
of (TPDD) by rotating the screw 360°, causing the screw to
increase the height of one side of the device by 1 mm. The pro-
cess was repeated in the following 2 days. Following periosteal
distraction, animals were sacrificed after 2 weeks in Group 1
and after 4 weeks in Group 2.

2.4. Surgical procedures

All surgeries were conducted under sterile conditions. Before
surgery, each rat was intramuscularly injected with 35 mg/kg
ketamine hydrochloride and 5 mg/kg xylazine hydrochloride
(Sanky, Tokyo, Japan). The cranial dorsal aspect was
shaved and aseptically prepared for surgery. A 10-mm-long
incision in the skin was made coronally on the posterior aspect
of the calvarial bone followed by periosteal incision. A sharp
periosteal elevator marked at 16 mm starting from its pointed
end was inserted in the incision site and directed anteriorly for
16 mm to raise the periosteum carefully from the bone surface creating a subperiosteal pouch where the distraction device was immediately inserted. The periosteal incision was then sutured with 3–0 silk suture (Foosin Medical, China) followed by closure of the skin incision (Fig. 1B).

Animals were observed for any complications following surgery. Subsequently, 15 mg/kg of oxytetracycline and 1.5 mg/kg of diclofenac sodium were injected, then every 24 h for the following 3 days.

2.5. Specimen preparation

Animals were sacrificed using a pentobarbital I.V. overdose (Narkorens, Meral GmbH, Hallbergmoos, Germany). The area of the augmented soft tissue was removed from the animal’s calvarium bone en-bloc along with the surrounding tissues then fixed in 10% neutralized formalin for 1 week. Random micro-computed tomography (micro CT) scanning of two specimens was done (SMX-90CT, Shimadzu, Kyoto, Japan); one specimen from each group. These two specimens were plastic-embedded using Technovit 7200 Heraeus Kulzer GmbH, Wehrheim, Germany) with the distractor device (TPDD) in place.

In the rest of the specimens (n = 9 for each time point), (TPDD) was carefully dissected from the surrounding soft tissue using a sharp blade. The generated soft tissue was then peeled from the underlying calvarial bone using a sharp periosteal elevator. The samples were then embedded in paraffin, stained with AZAN stain and observed under an optical microscope (BZ-8000, Keyence, Osaka, Japan).

2.6. Histomorphometry

For each histological sample, three coronal sections passing through the center of the device hole were analyzed (n = 27 sections per group) using ImageJ software 1.5b (NIH, Bethesda, MD, USA). Histomorphometric measurements were done to assess each new periosteal (NPt) layer thickness (NPt) and connective tissue layer thickness (CTt) (Fig. 2A). The number (Bv) of new blood vessels per square mm and their percentage area (Bv %) of the new connective tissue layer (CTt) were also calculated.

2.7. Statistics

Histomorphometric measurements were collected and described using range, mean, standard deviation, and median (minimum and maximum). Student’s t-test was used to assess the differences between the two groups. Analysis was done using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The significance level was p < 0.05.

3. Results

3.1. Post-operative recovery

After the surgery, all animals showed an uncomplicated recovery. Soft tissue healing was uneventful, without inflammation or swelling. All devices remained concealed under the soft tissue over the calvarial area throughout the experiment period without exposure. The micro CT scanning of two specimens revealed new bone formation that was significantly different (t = 4.791, p < 0.0001) at 2 weeks (0.41 ± 0.065 mm) compared with 4 weeks (0.51 ± 0.067 mm) only in areas close to the stable end of the (TPDD) (Fig. 2B).

3.2. Histological evaluation

3.2.1. Un-decalcified sections

Coronal sections of the resin-embedded calvarial tissue specimens showed the titanium mesh supporting the overlying skin tissue. Bone tissue regeneration was observed in both groups only over the original bone close to the un-elevated end of the device. Soft tissue ingrowth formed a layer above the new bone under the distraction device (Fig. 3).
Fig. 2  (A) Schematic presentation of the device after activation. *S*: the elevation screw, *T*: Titanium device, *NPt*: New periosteum thickness, *CTt*: connective tissue thickness, *OP*: old periosteum, *OB*: old bone, *NB*: new bone. The full elevation height is 3 mm and 1.8 mm is the height in the middle of the device. (B) Newly formed bone underneath device in transverse of micro-computed tomography view (red arrow).

Fig. 3  Un-decalcified histologic presentation of the coronal section in the distracted calvarium near to the stable end of the device. After (A) 2 weeks and (B) 4 weeks, a small layer of newly-generated bone lay above the original bone. A thick layer of connective tissue covering the new bone was generated underneath the titanium mesh. (Plastic embedding A: toluidine blue; B: toluidine blue, bar = 1 mm). *T*: the boundary of titanium mesh hole, *NB*: new bone, *OB*: site of the original bone.
3.2.2. Paraffin-embedded AZAN stained sections

3.2.2.1. Group 1 (2 weeks). The distraction space was fully occupied with the newly formed soft tissue fibroblast cells. At the bottom, a new periosteum composed of layers of collagenous fibers running parallel to the original calvaria bone surface was observed. Numerous venules and arterioles (red arrows in Fig. 4) were found within the tissue together with fat tissues (yellow arrows in Fig. 4).

The new periosteum was covered with a hypervascular loose connective tissue layer with scattered inflammatory cells in the space beneath the lower surface of the (TPDD). The upper surface of (TPDD) was covered with the original periosteaum and the overlying skin layers (Fig. 4).

3.2.2.2. Group 2 (4 weeks). The generated tissue showed similar histologic findings to Group 1, but with less thickness. However, the collagenous fibrous layer running parallel to the original bone surface showed a coarser pattern with wider vascularization. The overlying connective tissue layer showed less vascularization with more organized connective tissue fibers (Fig. 5).

3.3. Histomorphometry

A significant difference was observed between the new periosteum thickness at 2 weeks (0.89 ± 0.05 mm) and at 4 weeks (0.82 ± 0.07 mm) (p = 0.001). Also, a significant difference was observed in the number and percentage of blood vessels between Group 1 (155 ± 11.3; 0.22 ± 0.01%) and Group 2 (97.7 ± 13.8; 0.17 ± 0.03%) (p < 0.001). No significant difference was observed between the connective tissue thickness at the two time points (1.48 ± 0.14 mm and 1.42 ± 0.06 mm, p = 0.113) (Table 1).

4. Discussion

Most of the previous periosteal distraction studies (Casap et al., 2008; Kessler et al., 2007; Sato et al., 2010; Schmidt et al., 2002) used the calvarium model in different animal models to study the effect of the technique. In this preliminary study, we evaluated the ability of periosteal distraction, at a high rate of 1 mm/day, to generate vascularized connective tissue in the newly created space.

The regenerated tissue was assessed histologically and histomorphometrically at 2 weeks (Group 1) and 4 weeks (Group 2). In both groups, new periosteum was regenerated and covered the original bone surface in the distraction site. The distracted spaces showed a predominance of hyper-vascularized connective tissue. Group 2 showed more organized collagen fibers with less vascularity compared to Group 1. Micro CT scanning of specimens from two animals revealed little new bone formation near to the stable end of the device in both Group 1 (0.41 ± 0.065 mm) and Group 2 (0.51 ± 0.067 mm).

In this experiment, we used a distracting device to maintain this un-secluded space and so encourage soft tissue regeneration inside it. Not using such a mechanical barrier to maintain this newly created space would result in the collapse of the overlying soft tissue and loss of space and hence no connective tissue would be generated.

In this study, to prevent exposure of the soft tissue and periosteum overlying the new space created, we implemented a distraction rate of 1 mm/day for 3 days. Thus, we could keep the integrity of the overlying periosteum and preserve the wound edges without any disruption throughout the healing period.

Soft tissue was successfully regenerated in the space underneath the distraction device after the gradual elevation of the
periosteum. The distractor device was designed to create a space of an average volume of 188 mm$^3$ on the elevation of one side by 3 mm. Previous periosteal distraction device designs were characterized by multiple small holes; however, in this study, the device used included only two wide holes exposing almost 20% of the surface area of the overlying periosteum to the underlying space (Saulacic et al., 2012, 2013b).

Previous periosteal distraction latency periods ranged from 0 to 14 days with shorter periods being related to unfavorable osteogenesis and more soft tissue formation (Zhao et al., 2016). In this study, immediate activation of the device was performed by elevating the device 1 mm immediately after surgery. A coronal incision line for device placement lay totally outside the distraction area. Thus, immediate activation of the device did not cause the flap sutures to disrupt. Previous periosteal distraction studies used the mid-sagittal incision directly over the distraction area to place the device (Estrada et al., 2007; Zakaria et al., 2012a, 2012b). Consequently, immediate activation could jeopardize the overlying flap sutures.

Previous periosteal distraction studies recommended a speed of 0.25 mm/day to regenerate bone in the gap (Estrada et al., 2007). Other studies suggested an optimal distraction speed of 0.33 mm/day or lower. A high rate of distraction has been related to reduced bone quality and increased connective tissue regeneration in the distraction space (Zakaria et al., 2012c). This result was confirmed in the present study. Activating the device from one side enabled study of the effect of different periosteal distraction rates. The tissue regenerated within the created gap varied according to the elevation rate of the device in that region, where bone regeneration was confined to the region of low rate near the stable end. The remaining part of the distraction gap characterized by a higher periosteal distraction rate was occupied with connective tissue.

Previous studies that used the same surgical site and animal model as in the current study reported bone tissue regeneration (Saulacic et al., 2012, 2013a, 2013b). Activation of bone surface or perforating the cortical layer of bone has been shown to increase bone regeneration (Nakahara et al., 2017; Oda et al., 2009). In this study, no cortical perforation was done, and the cortical bone surface was kept intact during the reflec-

### Table 1: The number and percentage area of blood vessels per mm$^2$ in newly formed connective tissue.

| Parameter | 2 weeks (Group 1) | 4 weeks (Group 2) | $t$ | $p$-value |
|-----------|------------------|------------------|----|-----------|
| NP$_t$    | 0.895 ± 0.0573 mm| 0.822 ± 0.0702 mm| -3.603 | 0.0009* |
| CT$_t$    | 1.4864 ± 0.149 mm| 1.4269 ± 0.0693 mm| -1.619 | 0.1137   |
| BV$_n$    | 155 ± 11.38      | 97.725 ± 13.89    | -14.445 | < 0.0001* |
| BV$_n$ %  | 0.227 ± 0.01%    | 0.17 ± 0.03%      | -8.061  | < 0.0001* |

Values are presented as mean ± standard deviation.

NP$_t$: new periosteum thickness
CT$_t$: connective tissue thickness
BV$_n$: number of blood vessels in connective tissue per mm$^2$
BV$_n$ %: percentage of blood vessels in connective tissue

* Statistically significant difference between Groups 1 and 2.
tion of the mucoperiosteum to minimize the release of mesenchymal stem cells and angioblasts from the underlying cancellous bone layer.

In this study, a barrier membrane was not used, to allow the connective tissue cells to occupy the newly created space—unlike the principle of guided bone regeneration (Dahlin et al., 1989). The distraction space in both groups was mostly occupied with vascular connective tissue, a 1.48 mm thickness at the 2-week time point and 1.42 mm at the 4-week time point. Its height represented 82% of the total distracted height (1.8 mm) at 2 weeks (Group 1) while it was almost 78% at 4 weeks (Group 2). These results show keeping the distracted space open for a longer duration was not associated with increased soft tissue thickness. This decrease in soft tissue regeneration may be due to the different organization of tissue at the 4-week time point compared with that at 2 weeks. The generated connective tissue was abundant with blood vessels with an average count of 155 per mm² at 2 weeks (Group 1) decreasing to 97 per mm² at 4 weeks (Group 2). The bottom layer of the generated tissue constituted a typical periosteum that occupied about 30% of the generated tissue height in both groups.

The main limitation of this study is the short observation period for the generated connective tissues. Also, the study did not demonstrate precisely if the regenerated tissue characterized by reduced bone formation and generous vascular connective tissue was due to a shortened latency period, high periosteal distraction rate, or modified device design, or due to the combined effect of all these factors. The addition of a control group to this study could have strengthened the results of this study. However, we report that periosteal distraction using 1 mm/day in rat calvarial model has effectively created abundant and vascular connective tissue in situ in addition to the creation of new periosteum above the original bone. Applying this technique clinically may represent a minimally invasive alternative method to the harvesting of autogenous palatal connective tissue grafts to treat patients with aesthetic soft tissue challenges. Clinically, the soft tissue defects after extraction procedures are more prevalent at buccal and labial sites than palatal. So, in situ augmentation of the labial or buccal intraoral defects would be more rational. However, applying such procedures in the labial or buccal sites in small animals is not feasible mainly due to space limitation, in addition to interference with the feeding of the animals. So future studies using this technique in a bigger animal model such as a canine or goat model for buccal defect augmentation are recommended.

We recommend that future studies comparing periosteal distraction and periosteal tenting should be carried out with a longer follow-up period. Also, comparing the effect of removing or keeping the periosteum (shielding the periosteum using a membrane) should be further evaluated. Use of immunohistochemistry in future studies may also give a more detailed evaluation of the regenerated tissues.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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