Periodontal tissue regeneration with PRP incorporated gelatin hydrogel sponges

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Abstract
Gelatin hydrogels have been designed and prepared for the controlled release of the transforming growth factor (TGF-b1) and the platelet-derived growth factor (PDGF-BB). PRP (Platelet rich plasma) contains many growth factors including the PDGF and TGF-b1. The objective of this study was to evaluate the regeneration of periodontal tissue following the controlled release of growth factors in PRP. For the periodontal ligament cells and osteoblast, PRP of different concentrations was added. The assessment of DNA, mitochondrial activity and ALP activity were measured. To evaluate the TGF-b1 release from PRP incorporated gelatin sponge, amounts of TGF-b1 in each supernatant sample were determined by the ELISA. Transplantation experiments to prepare a bone defect in a rat alveolar bone were an implanted gelatin sponge incorporated with different concentration PRP. In DNA assay and MTT assay, after the addition of PRP to the periodontal ligament cells and osteoblast, the cell count and mitochondrial activity had increased the most in the group with the addition of 5 × PRP. In the ALP assay, after the addition of PRP to the periodontal ligament cells, the cell activity had increased the most in the group with the addition of 3 × PRP. In the transplantation, the size of the bone regenerated in the defect with 3 × PRP incorporated gelatin sponge was larger than that of the other group.

1. Introduction
Periodontal disease is a chronic inflammatory disease caused by bacteria in plaque. In periodontal disease, the destruction of collagen fibers, the absorption of alveolar bone and an increase of periodontal pockets occur depending on the progress of the conditions [1] and the teeth are eventually lost in some cases. If inflammation of the periodontal tissue remains after initial periodontal treatment focused on plaque control, periodontal flap surgery, or the guided tissue regeneration (GTR) or enamel matrix derivative (EMD), depending on the conditions of lost tissue, may be used for tissue regeneration [2]. However, currently only cases with bone defects of the double or triple vertical walls are eligible for periodontal tissue regeneration using GTR or EMD, whereas in cases with horizontal bone defects, alveolar bone regeneration is insufficient [2]. Therefore, to regenerate bone in the defective area, the application of a new regeneration method in addition to the regeneration of the existing bone is necessary.

Tissue regeneration is known to involve three elements of cell, scaffold and cell growth factors. To effectively extract the physiological effects of cell growth factors, it is necessary to maintain the cell growth factors localized for an appropriate time period or to use carriers for controlled release at an appropriate rate [3]. A drug delivery system (DDS) is one plausible technology for enhancing the in vivo biological activity of growth factors [4].

Bio-degradable gelatin hydrogels have been designed and prepared for the controlled release of bioactive basic fibroblast growth factor (b-FGF) [5], transforming growth factor beta 1 (TGF-b1) [6], platelet-derived growth factor BB (PDGF-BB) [7] and bone morphogenetic protein 2 (BMP-2) [8]. Asamura et al [8] reported that the regeneration of new bone was promoted in a dog model of orbital floor fracture through the controlled release of BMP-2 from gelatin hydrogel. Platelet growth factors (PGF) can also be released from gelatin hydrogel, demonstrating their enhanced activity to induce angiogenesis [7], or regenerate knee meniscus [9], adipogenesis [10] and wound healing [11].

Platelet rich plasma (PRP) is a fraction rich in platelets. PRP can be produced by autologous blood, which contains many cell growth factors including the PDGF, TGF-b1, vascular endothelial growth factor (VEGF)
prepared platelets were mixed with CaCl₂ solution at the platelets for the release of growth factors, the in 1.0 ml of plasma was used as the PRP. To activate platelet pellet was collected and a thrombolytic pellet and then centrifuged for 5 min at 1600 G and 4°C. The platelet pellet was collected and a thrombolytic pellet in 1.0 ml of plasma was used as the PRP. To activate the platelets for the release of growth factors, the prepared platelets were mixed with CaCl₂ solution at concentrations of 2 wt% at a ratio of 7:1 by volume, and then centrifuged for 5 min at 1600 G and 4°C. The supernatant solution was used as the activated PRP. All the animal experiments were approved by the Kyoto University Committee for Animal Experimentation (Authorization No. F-140).

2.1.1. Assessment of platelet counts in PRP
We measured the platelet count in the PRP and the platelet count in the whole blood for comparison. Samples containing triple the platelet count in the PRP than in the whole blood were triple concentrated PRP, and those with five times the platelet count were five-times concentrated PRP. We used platelet poor plasma (PPP) to dilute the PRP.

2.1.2. Assessment of cell growth factors in PRP
Concentrations of the cell growth factors (TGF-β1 and PDGF-BB) in the PRP were measured using the Elisa Kit (Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, MN, USA)).

2.2. Examination of PRP efficacy for cells
2.2.1. Cells
2.2.1.1. Periodontal ligament cells For the rat periodontal ligament cells used in the experiments, we used cells grown with the outgrowth method. Briefly, the maxillary molar (M1, 2) was removed from four F344 male 4 week-old rats (Shimizu Laboratory Supplies, Co Ltd, Kyoto, Japan) and static culture was executed on the tooth for 10 d in MEM α medium (Sigma-Aldrich, CA, USA) containing 15% fetal bovine serum (FBS) (JR Scientific, CA, USA), antibiotics (100 U ml⁻¹ Penicillin G and 100 μg ml⁻¹ streptomycin) (Gibco Life Technologies, CA, USA), and antifungitics (250 ng ml⁻¹ amphotericin B) (Gibco Life Technologies, CA, USA) in six wells dishes. To confirm the bone differentiation potency of the grown cells, they were cultivated in a bone differentiation-inducing medium. The bone differentiation induction medium contained 15% FBS, 100 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 mM glycercophosphoric acid and 50 μM ascorbic acid. The culture medium was replaced every three days. Alizarin red dye coloring was implemented 21 d after starting the culture. The grown cells were confirmed by coloration with alizarin dye, so these cells were periodontal ligament cells (figure 1). Cells that underwent 3–5 passages were used in this study.

2.2.1.2. Osteoblasts The MC3T3-E1 was purchased from Sigma-Aldrich (USA). The cell culture was performed with MEM α medium containing 10% FBS (JR Scientific, CA, USA), antimicrobial agent (100 U ml⁻¹ Penicillin G and 100 μg ml⁻¹ streptomycin) (Gibco Life Technologies, CA, USA), and antifungitics (250 ng ml⁻¹ amphotericin B) (Gibco Life Technologies, CA, USA).

2.2.2. Effect of PRP on cells
2.2.2.1. DNA assay Periodontal ligament cells and osteoblasts suspensions were each prepared at 5.0 × 10⁶ cells ml⁻¹. The prepared cell suspension (1 ml) was distributed into six wells dishes. After 24 h of static culture, the culture medium was exchanged with 1 ml of new culture medium. In the medium, PRP of different concentrations (single concentration (1×), triple concentration (3×) or quintuple concentration (5×)) were added, and the PRP content in the culture medium was prepared at 1, 5 or 10 wt%. The culture medium exchange and PRP addition took place once every three days. An assessment of the cell DNA count took place after 24, 48, 72 and 168 h of cultivation for the periodontal ligament cells and osteoblasts, respectively. For the assessment, 500 μl
of sodium dodecyl sulfate (SDS) was added to the cells and the cell lysate was collected. Next, 100 μl of the collected cell lysate was added to a 96 wells clear plate. Hoechst solution (Dojindo Molecular Technologies, Inc, Japan) was prepared by adding 10 ml of standard saline citrate (SSC) to 10 μl of Hoechst. To the cell lysate, 100 μl of Hoechst solution was added. The DNA content was measured using a fluorescence microplate reader (Versa Max, Molecular Device Japan Ltd, Tokyo, Japan) (λ: 405 nm). The controls were periodontal ligament cells and osteoblasts cultured with a MEM α medium containing 10% FBS, respectively.

2.2.2.2. MTT assay The periodontal ligament cells and osteoblasts suspensions were each prepared at 1.0 × 10^5 cells ml^-1. The prepared cell suspension (100 μl) was distributed into a 96 wells dish. After 24 h of static culture, the culture medium was exchanged with 1 ml of new culture medium. In the medium, PRP of different concentrations (1×, 3× or 5×) was added, and the PRP content in the culture medium was prepared at 1, 5 or 10 wt%. An assessment of the mitochondrial activity was measured using Cell Count Reagent SF (nacalai, Kyoto, Japan) and took place after 24, 48 and 72 h of cultivation for the periodontal ligament cells and osteoblasts, respectively. For the assessment, 100 μl of WST-8 solution (Nacalai, Kyoto, Japan) (λ: 450 nm) (n = 5). The controls were periodontal ligament cells and osteoblasts cultured with a MEM α medium containing 10% FBS, respectively.

2.2.2.3. ALP assay An assessment of the ALP activity was measured by ALP Kit (Wako, Osaka, Japan). The periodontal ligament cells and osteoblasts suspensions were each prepared at 5.0 × 10^4 cells ml^-1. The prepared cell suspension (1 ml) was distributed into six wells dishes. After 24 h of static culture, the culture medium was exchanged with 1 ml of new culture medium. In the medium, PRP of different concentrations (1×, 3× or 5×) was added, and the PRP content in the culture medium was prepared at 1, 5 or 10 wt%. An assessment of the ALP activity was measured by adding sodium p-nitrophenylphosphate tablet to the buffer solution. To the cell lysate, 100 μl of substrate buffer was added and incubated at 37°C for 15 min. Next, 80 μl of 0.02 N NaOH solution was added and the absorbance intensity was measured using an absorbance microplate reader (Versa Max, Molecular Device Japan Ltd, Tokyo, Japan) (λ: 405 nm). The controls were periodontal ligament cells and osteoblasts cultured with the bone differentiation-inducing medium.

2.3. Preparation of gelatin sponges A gelatin sample with an isoelectric point (IEP) of 5.0 was prepared through an acidic process of porcine skin collagen type I (Nitta Gelatin Co., Osaka, Japan). β-tricalcium phosphate (β-TCP) granules (2 μm on average in diameter) were obtained from Taihei Chemical Industries (Nara, Japan). Glutaraldehyde (GA), glycine and other chemicals were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Bio-degradable gelatin hydrogel sponges were prepared by the glutaraldehyde (GA) (Wako, Pure Chemical Industries, Osaka, Japan) cross-linking of an aqueous gelatin solution. Briefly, 80 μl of aqueous GA solution (25 wt%) with 40 g of aqueous gelatin solution (5 wt%) was preheated at 40°C. The preheated solution was mixed for 15 s by the homogenizer, after the addition of 10 wt% of β-TCP. The mixed aqueous solution was cast into a polypropylene dish (138 mm × 138 mm and 5 mm depth, Thermo Fisher Scientific Inc, MA, USA) followed by being left at 4°C for 12 h for gelatin cross-linking. Then, the cross-linked gelatin hydrogel sponges with β-TCP were placed into 100 mM of aqueous glycine solution at 37°C for 1 h to block the residual aldehyde groups of glutaraldehyde. Following complete washing with double distilled water (DDW), the gelatin hydrogel sponges were freeze-dried.

2.4. In vivo experiments

2.4.1. Animals For the in vivo experiments, fifty-six rats (F344, male, 8 weeks old: Shimizu Laboratory Animal Supply, Kyoto, Japan) were used.

2.4.2. Preparation of alveolar bone defects in rats The alveolar bone defects were prepared under intraperitoneal anesthesia of the rats with pentobarbital sodium (0.65 mg kg^-1). The mouths were opened with the animals restrained in a reclining position. Then, an intracrevicular incision was made with a No. 12 substitute scalpel (Feather Safety Razor, Osaka, Japan) from the palatal mesial angle of the upper left second molar (M2) to the mesial center of the upper left first molar (M1), and the incision was extended along the alveolar crest in the mesial direction. After the incision, flap reflection with a full-thickness flap was performed using a dental excavator, and the alveolar bone, periodontal ligament and cementum were excised using a dental round bur (ISO Standard 010) under physiological saline irrigation to prepare the alveolar bone defect (width × length × distance; 2 mm × 2 mm × 2 mm) according to the method of Yu et al[14] (figure 2).
2.4.3. Preparation of PRP incorporated gelatin hydrogel sponges
The experiment groups underwent an implantation of (a) PRP (1×) incorporated gelatin hydrogel sponges, (b) PRP (3×) incorporated gelatin hydrogel sponges, (c) PRP (5×) incorporated gelatin hydrogel sponges (d) PRP (1×), (e) PRP (3×), (f) PRP (5×), (g) gelatin sponges and (h) non-implant. The PRP incorporated gelatin hydrogel sponges were prepared by PRP (3 μl) impregnated into a gelatin sponge (300 μg), followed by being left at 4°C overnight.

2.5. Histological examination
Two and four weeks after implantation, the rats were put under general anesthesia. The carotid was cut, and cervical dislocation was performed. Then, the maxilla including the periodontal tissue was excised and fixed with 10% neutral phosphate-buffered formalin. After completion of fixation, decalcification was performed for six weeks using 10% EDTA solution, and embedded in paraffin according to the usual method. Subsequently, 5-μm-thick serial specimens were prepared in the mesial–distal plane. Finally, hematoxylin–eosin (HE) stain was used for observation under an optical microscope (AX80 Provis, Olympus Ltd, Tokyo, Japan). This area measurement was subsequently normalized by the size of the total defect area. The part of the connective tissue fibers of the root that adhered vertically was measured as a range of attachment of the connective tissue (figure 2).

2.6. Statistical analysis
All the statistical data were expressed as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance followed by the Tukey test. Values of p less than 0.05 were considered to be significant.

3. Results

3.1. Evaluations of PRP
3.1.1. Evaluation of the number of platelets in the PRP
About 5.6 × 10^6 platelets ml⁻¹ were included in the whole blood and 4.7 × 10^9 platelets ml⁻¹ in PRP, respectively (figure 3).

3.1.2. Evaluation of growth factors in the PRP
The concentrations of TGF-β1 were 33.0 ± 0.9 ng ml⁻¹ in the whole blood, 37.7 ± 3.4 ng ml⁻¹ in PRP (1×), 103.9 ± 1.7 ng ml⁻¹ in PRP (3×) and 134.8 ± 2.4 ng ml⁻¹ in PRP (5×) (figure 4(a)). The concentrations of PDGF-BB were 1.9 ± 0.9 ng ml⁻¹ in the whole blood, 15.0 ± 1.1 ng ml⁻¹ in PRP (1×), 24.4 ± 0.6 ng ml⁻¹ in PRP (3×) and 46.1 ± 3.5 ng ml⁻¹ in PRP (5×) (figure 4(b)).

3.2. Examination of PRP efficacy for cells
3.2.1. DNA assay
3.2.1.1. Periodontal ligament cells
At 24, 48 and 72h after the addition of PRP, no significant differences were seen in the periodontal ligament cell count among the groups (p > 0.05) (figures 5(a)–(c)). At 168h after the addition of PRP, the periodontal ligament cell count had the largest number in the group with PRP (5×) addition, when the PRP content was 5 or 10wt% (p < 0.05) (figure 5(d)).

3.2.1.2. Osteoblasts
At 24h after the addition of PRP, no significant differences were seen in the cell count between the groups (figure 6(a)). At 48h after the addition of PRP, the cell count had the largest number in the group with PRP (5×) addition, when the PRP content was 1 or 5 wt% (p < 0.05). A significant difference in the cell count was found between the group with PRP (5×) addition and the group with PRP (3×) addition, as well as between the group with the addition of PRP (1×) and the group with PRP (3×) addition, when the PRP content was 10 wt% (p < 0.05) (figure 6(b)). At 72h after the addition of PRP, the cell count had the largest number in the group with PRP (5×) addition when the PRP content was 5 or 10 wt% (p < 0.05) (figure 6(c)). At 168h after the addition of PRP, the cell count had the largest number in the group with PRP (5×) addition when the PRP content was 5 or 10 wt% (p < 0.05). No significant difference in the cell count was found between the group with PRP (1×) addition and the group with the addition of PRP (3×) content of 5 or 10 wt% (p > 0.05) (figure 6(d)).

3.2.2. MTT assay
3.2.2.1. Periodontal ligament cells
At 24h after the addition of PRP, the mitochondrial activity showed a concentration-dependent increase, when the PRP content was 5 or 10 wt% (p < 0.05) (figure 7(a)). At 48h after the addition of PRP, the mitochondrial activity showed a concentration-dependent increase, regardless of the PRP content in the culture medium (p < 0.05)
At 72 h after the addition of PRP, the mitochondrial activity showed a concentration-dependent increase, when the PRP content was 5 or 10 wt% ($p < 0.05$). A significant difference in the mitochondrial activity was found between the group with PRP (5×) addition and the group with PRP (1×) addition when the PRP content was 1 wt% ($p < 0.05$) (figure 7(c)).

A significant difference in the mitochondrial activity was found between the group with PRP (3×) addition and the group with PRP (1×) addition, when the PRP content was 5 wt% ($p < 0.05$). The mitochondrial activity had the largest number in the group after the addition of PRP. A significant difference in the mitochondrial activity was found between the group with PRP (5×) addition and the group with PRP (1×) addition, as well as between the group with PRP (3×) addition and the group with PRP (1×) addition, when the PRP content was 1 wt% ($p < 0.05$).

### 3.2.2. Osteoblasts

At 24 h after the addition of PRP, no significant differences were seen in the mitochondrial activity between the groups (figure 8(a)). At 48 h after the addition of PRP, a significant difference in the mitochondrial activity was found between the group with PRP (5×) addition and the group with PRP (1×) addition, when the PRP content was 5 wt% ($p < 0.05$). The mitochondrial activity had the largest number in the group...
with the addition of PRP (5×) when the PRP content was 10 wt% (p < 0.05) (figure 8(b)). At 72 h after the addition of PRP, the mitochondrial activity showed a concentration-dependent increase, when the PRP content was 1 or 5 wt% (p < 0.05). The mitochondrial activity had the largest number in the group with the addition of PRP (5×), when the PRP content was 10 wt% (p < 0.05) (figure 8(c)).
Figure 6. DNA assay for osteoblast. (a) At 24 h after the addition of PRP. (b) At 48 h after the addition of PRP. (c) At 72 h after the addition of PRP. (d) At 168 h after the addition of PRP. Control: osteoblast cultured with a MEM α basic medium containing 10% FBS. *$p < 0.05$, $^*p < 0.05$; statistically significant difference when compared with the osteoblast cultured with a MEM α basic medium containing 10% FBS.
3.2.3. ALP assay

3.2.3.1. Periodontal ligament cells  At 24 h after the addition of PRP, the group with PRP (3×) addition had the largest number in ALP activity compared with the other groups when the PRP content was 5 or 10 wt% \( (p < 0.05) \). A significant difference in ALP activity was found between the group with PRP (3×) addition and the group with PRP (5×) addition, when the PRP content was 1 wt% \( (p < 0.05) \) (figure 9(a)). At 48 h after the addition of PRP, the group with PRP (3×) addition had the largest number in ALP activity compared with the other groups, when the PRP content was 5 or 10 wt% \( (p < 0.05) \) (figure 9(b)).

3.2.3.2. Osteoblasts  At 168 and 336 h after the addition of PRP, no significant differences were seen in ALP activity between the groups \( (p > 0.05) \) (figures 10(a) and (b)).

3.3. Histological observations

3.3.1. Two weeks after implantation

Figure 11 shows the representative histological images at the alveolar bone deficiency and figure 12 shows the areas (μm²) of bone regeneration for each group 2 weeks after implantation, respectively. In all the groups, bone regeneration was observed. The group with PRP (5×) incorporated gelatin hydrogel sponges and the group
with PRP (1×) regenerated the bone compared with the non-implant group \( (p < 0.05) \). Inflammatory cell infiltration was confirmed in the part corresponding to the gelatin sponge-implanted part in the gelatin sponge-implanted groups.

### 3.3.2. Four weeks after implantation
Figure 13 shows the representative histological images at the alveolar bone deficiency. Figure 14 shows the area (\( \mu m^2 \)) at bone regeneration. Figure 15 shows the length (\( \mu m \)) of the attachment connective tissue for each group 4 weeks after implantation, respectively. From the histological sections, the most bone regeneration was observed in the group with PRP (3×) incorporated gelatin hydrogel sponges compared with the other groups \( (p < 0.05) \). The group with PRP (5×) incorporated gelatin hydrogel sponges regenerated the bone compared with the group with PRP (3×), the group with PRP (5×), the group with gelatin sponges, the group with PRP (1×) incorporated gelatin hydrogel sponges and the non-implant group (figure 14). The group with PRP (5×) incorporated gelatin hydrogel sponges and the group with gelatin sponges had increased attachment connective tissue compared with the groups with PRP (1×), PRP (3×) and PRP (5×) (figure 15).

### 4. Discussion
PRP is a fraction containing a large number of platelets. PRP contains not only TGF-\( \beta \) and PDGF but also
fibronectin, fibrinogen and vitronectin. Cell growth factors and adhesion factors promote wound healing and the regeneration of periodontal tissues [13, 15, 16]. Currently, there are expectations for PRP application in the dental field as filling bone deficiency in periodontal surgery [16], maxillary sinus floor elevation [18], alveoplasty [19] and implantation [20].

In this experiment, we first examined changes in cell count, mitochondrial activity and ALP activity under in vitro conditions in periodontal ligament cells and osteoblasts with added PRP according to the degree of concentration and content. At 168 h after the addition of PRP, the periodontal ligament cell count had the largest number in the group with the addition of PRP (5×).

Han et al [21] reported that as the TGF-β1 concentration increased, the cell count in the periodontal ligament cells also increased. TGF-β1 has growth-promoting activity for mesenchymal cells, such as fibroblasts, as well as expression/production-promoting activity on an extracellular matrix, such as collagen, fibronectin and laminin. In addition, Han et al [21] reported an increase in ALP activity in periodontal ligament cells in a culture medium up to a TGF-β1 concentration of 100 ng ml⁻¹ and decreased ALP activity in periodontal ligament cells when the TGF-β1 concentration exceeded 100 ng ml⁻¹. In this study, ALP activity was the highest in the group with a TGF-β1 concentration of 103.9 ng ml⁻¹ with PRP (3×) added to the medium. Furthermore, the lowest ALP activity was observed in the group with a TGF-β1 level of 134.8 ng ml⁻¹ contained in PRP with PRP (5×) added to the medium. Thus, it can be hypothesized that TGF-β1 has a function of suppressing ALP activity in periodontal ligament cells above a certain concentration. Moreover, Suzuki et al [22] reported that when BMP-2 is added to periodontal ligament cells, the ALP activity in these cells decreases. Therefore, it can be hypothesized that PRP (3×) to PRP (5×) preparations contain TGF-β1 and BMP-2 at concentrations suppressive to ALP activity in periodontal ligament cells. After 336 h of cultivation, the ALP activity in the osteoblasts tended to increase more than after 168 h of cultivation. Goto et al [23]
reported that ALP staining was confirmed at 21 d after the addition of PRP. The ALP activity of osteoblasts is considered to be elevated late with the addition of PRP.

In tissue engineering, carriers to place a 3D mold made from bio-absorbable materials on deficient parts to arrange cells three-dimensionally promoting the attachment, growth and differentiation of cells to induce the regeneration of body tissues are considered to be important [4]. In periodontal tissue regeneration therapy, especially in cases with wide bone deficiency, such as single wall and horizontal cases, it is difficult to induce the regeneration of tissue. The gelatin sponge used in this study can be readily fabricated in sponge, sheet and particle forms and also has the flexibility to fit deficient formations [7, 24, 25]. Furthermore, PRP gelatin was used in a rat ulna fracture model in which the deficiency was comparatively large and regeneration induction was difficult, and it was reported to be effective in bone regeneration [26].

PRP contains various cell growth factors. The PDGF acts as a cell mitogen in osteoblasts, endothelial cells and mesenchymal stem cells. The TGF-β promotes cell division, angiogenesis and the differentiation of mesenchymal stem cells to osteoblasts. VEGF adds growth of capillaries. Cell growth factors are not necessarily expected to exert biological activity in vivo, due to rapid enzymatic digestion and the inactivation of cell growth factors [6]. Therefore, delivery technology to effectively promote the bioactivity of cell growth factors is important. In gelatin hydrogel systems, cell growth factors that are under controlled release interact physicochemically with gelatin particles [7]. The cell growth factors are thus emitted from the hydrogel through the water solubilization of the gelatin particles that occurs as the hydrogel degrades.

The wound-healing mechanism during a bone transplant starts to show the division of osteogenic cells and the formation of capillaries three days after transplant; the permeation of the capillaries in the transplanted part is complete and the number of osteogenic cells markedly increases 17–21 d after transplant. The first stage of this type of bone transplant healing takes
place in the first three weeks after transplant; therefore, it is important to effectively deliver the cell growth factors to the deficiency during this first stage. The gelatin sponge we used this time is designed to be absorbed in vivo within two weeks. Therefore, it is suggested that cell growth factors are under controlled release during the first stage of healing.

In vitro experiments, periodontal ligament cells and osteoblasts tended to increase the cell count and mitochondrial activity when high concentration PRP was added. But the ALP activity in the periodontal ligament increased after the group with PRP (3×) was added. In the in vivo experiment four weeks after implantation, the group with PRP (3×) incorporated gelatin hydrogel sponges showed significantly more bone regeneration than the group with PRP (1×) incorporated gelatin hydrogel sponges, the group with PRP (5×) incorporated gelatin hydrogels sponges, the group with PRP alone and the group with gelatin sponges. These results suggest that there is an appropriate concentration or amount to allow cell growth factors to contribute to bone regeneration. Moreover, it is possible that the reason for the greatest bone regeneration tendencies being seen in the groups with PRP incorporated gelatin
sponges is that the cell growth factors were released in a sustained manner depending on the degradation of the gelatin sponge. In addition, no appreciable difference in bone regeneration was seen between the PRP concentration factors in the PRP alone groups. When PRP alone was implanted, the PRP flowed off the deficiency site and it may not have contributed to the regeneration process. Bone regeneration was seen in the group using a gelatin sponge independently. There is a possibility that the remaining cells in the deficiency and surrounding blood permeated the pores of the gelatin sponge and the cells matured therein. When comparing the gelatin sponge-implanted group and the non-implant group, the range of connective tissue attachment had a tendency to be bigger in the implanted group. The reason for the attachment range of the connective tissue being bigger in the group with PRP incorporated gelatin hydrogel sponges implant may inhibit the growth of epithelial cells by the TGF-β1 [27]. Furthermore, between the root of the tooth and the regenerated bone, fibers running vertically to the root of the tooth were found.

Figure 13. Histological images of bone regenerated in the periodontal defects of F344 at 4 weeks after implantation. (a) PRP (1×) + gelatin sponge, (b) PRP (3×) + gelatin sponge, (c) PRP (5×) + gelatin sponge, (d) gelatin sponge, †: regenerated bone, C: cementum, D: dentin, AB: alveolar bone, bars = 200 μm.

Figure 14. Area of bone regenerated in the periodontal defects of F344 at 4 weeks after implantation. *p < 0.05; statistically significant difference when compared with the PRP (3×) + sponge, #p < 0.05; statistically significant difference when compared with the PRP (5×) + sponge, §p < 0.05; statistically significant difference when compared with the non-implant group.
In this study, cell growth factors were under controlled release from gelatin and then we observed bone regeneration and the attachment of connective tissue.

5. Conclusion

We reached the following conclusions in this study by examining the effects of PRP on periodontal tissue regeneration.

1) At 168 h after the addition of PRP to the periodontal ligament cells, the cell count had increased the most in the group with PRP (5×) addition, when the PRP content was 5 or 10 wt% (p < 0.05).

2) At 24, 48 and 72 h after the addition of PRP to the periodontal ligament cells, the mitochondrial activity in the cells showed a concentration-dependent increase, when the PRP content was 5 or 10 wt% (p < 0.05).

3) At 24 and 48 h after the addition of PRP to the periodontal ligament cells, the group with PRP (3×) addition had the largest number in ALP activity compared with the other experimental groups when the PRP content was 5 or 10 wt% (p < 0.05).

4) At 72 and 168 h after the addition of PRP to the osteoblasts, the group PRP (5×) addition had the largest number in cell counts, when the PRP content was 5 or 10 wt% (p < 0.05).

5) At 72 h after the addition of PRP to the osteoblast, the group with PRP (5×) addition had the largest number in mitochondrial activity, regardless of the PRP content.

6) At 4 weeks after implantation, the new bone regeneration in the PRP (3×) incorporated gelatin sponge was more than that of the other groups.

7) At 4 weeks after implantation, the new attachment in the group implanted with gelatin sponges was more than that of the other groups.

From the above results, cell growth factors in PRP are effective in the periodontal regeneration process, which may be attributable to the sustained emission of cell growth factors.

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Figure 15. Lengths of attachment of connective tissue in the periodontal defects of F344 at 4 weeks after implantation. *p < 0.05. PRP.
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